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<b>(54) Title:</b> GLUTATHIONE-S-CONJUGATE TRANSPORT IN PLANTS  <b>(57) Abstract</b> <p>The invention includes an isolated DNA encoding a plant GS-X pump polypeptide and an isolated preparation of a plant GS-X pump polypeptide. Also included is an isolated preparation of a nucleic acid which is antisense in orientation to a portion or all of a plant GS-X pump gene. The invention also includes cells, vectors and transgenic plants having an isolated DNA encoding a plant GS-X pump and methods of use thereof. In addition, the invention relates to plant GS-X pump promoter sequences and the uses thereof.</p>		

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## GLUTATHIONE-S-CONJUGATE TRANSPORT IN PLANTS

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## BACKGROUND OF THE INVENTION

Animal and plant cells have the capacity to eliminate a diversity of lipophilic toxins from the cytosol following conjugation of the toxin with glutathione (GSH) (Ishikawa et al., 1997, *Bioscience Reports*. 17:189-208; Martinoia et al., 1993, *Nature* 364:247-249; Li et al., 1995, *Plant Physiol.* 107:1257-1268). This process is mediated by the glutathione S-conjugate (GS-X) pumps which are novel MgATP-dependent transporters that catalyze the efflux of GS-conjugates and glutathione disulfide (GSSG) from the cytosol via the plasma membrane and/or endomembranes. GS-X pumps are considered to constitute a terminal phase of xenobiotic detoxification in animals and plants.

The metabolism and detoxification of xenobiotics comprises three main phases (Ishikawa, 1992, *supra*). Phase I is a preparatory step in which toxins are oxidized, reduced or hydrolyzed to introduce or expose functional groups having an appropriate reactivity. Cytochrome P450 monooxygenases and mixed function oxidases are examples of phase I enzymes. In phase II, the activated derivative is

conjugated with GSH, glucuronic acid or glucose. In the case of the GSH-dependent pathway, S-conjugates of GSH are formed by cytosolic glutathione-S-transferases (GSTs). In the final phase, phase III, of the GSH-dependent pathway, GS-conjugates are eliminated from the cytosol by the GS-X pump.

5           The GS-X pump is unique in its exclusive use of MgATP, rather than preformed transmembrane ion gradients, as a direct energy source for organic solute transport. Although an understanding of the constituents of GS-X pumps is relevant to an understanding of the mechanism by which cells combat, for example, chemotherapeutic agents and herbicides, there has until recently been a paucity of  
10 information on the molecular identity of GS-X pumps, particularly in plants.

A 190 kDa membrane glycoprotein encoded by the human multidrug resistance-associated protein gene (*MRP1*) has been implicated in the resistance of small cell lung cancer cell lines to a number of chemotherapeutic drugs (Cole *et al.*, 1992, *Science* 258:1650-1654). This glycoprotein catalyzes the MgATP-dependent  
15 transport of leukotriene C<sub>4</sub> and related glutathione-S-conjugates (Leier *et al.*, 1994, *J. Biol. Chem.* 269:27807-27810; Muller *et al.*, 1994, *Proc. Natl. Acad. Sci. USA* 91:13033-13037; Zamam *et al.*, 1995, *Proc. Natl. Acad. Sci. USA* 92:7690-7694).

MRP1 is a member of the ATP binding cassette (ABC) superfamily of transporter proteins. Distributed throughout the major taxa, ABC transporters catalyze  
20 the MgATP-dependent transport of peptides, sugars, ions and lipophiles across membranes. ABC transporters comprise one or two copies each of two basic structural elements, a hydrophobic integral membrane sector containing approximately six transmembrane  $\alpha$  helices and a cytoplasmically oriented ATP-binding domain known as a nucleotide binding fold (NBF) (Hyde *et al.*, 1990, *Nature* 346:362-365; Higgins,  
25 1995, *Cell* 82:693-696). The NBFs are a diagnostic feature of ABC transporters and are 30% identical between family members over a span of about 200 amino acid residues, having two regions known as a Walker A and a Walker B box (Walker *et al.*, 1992, *EMBO J.* 1:945-951), and also having an ABC signature motif (Higgins, 1995, *supra*).



ABC family members in eukaryotes include mammalian P-glycoproteins (P-gps or MDRs), some of which are implicated in drug resistance and others in lipid translocation (Ruetz *et al.*, 1994, *Cell* 77:1071-1081), the pleiotropic drug resistance protein (PDR5) and STE6 peptide mating pheromone transporter of yeast, the cystic fibrosis transmembrane conductance regulator (CFTR) Cl<sup>-</sup> channel, the malarial *Plasmodium falciparum* chloroquine transporter (PFMDR1) and the major histocompatibility (MHC) transporters responsible for peptide translocation and antigen presentation (Balzi *et al.*, 1994, *J. Bioenerg. Biomemb.* 27:71-76; Higgins, 1995, *supra*).

Sequence comparisons between MRP1 and other ABC transporters reveal two major subgroups among these proteins (Cole *et al.*, 1992, *supra*; Szczypka *et al.*, 1994, *J. Biol. Chem.* 269:22853-22857). One subgroup comprises MRP1, the *Saccharomyces cerevisiae* cadmium factor (*YCF1*) gene, the *Leishmania* P-glycoprotein-related molecule (Lei/PgpA) and the CFTRs. The other subgroup comprises the multiple drug resistance proteins (MDRs), MHC transporters and STE6.

The invention described herein relates to bioremediation (specifically phytoremediation), plant responses to herbicides, plant-pathogen interactions and plant pigmentation.

With respect to bioremediation, the massive global expansion in industrial and mining activities during the last two decades together with changes in agricultural practices, has markedly increased contamination of groundwaters and soils with heavy metals. Indeed, it is estimated that the annual toxicity of metal emissions exceeds that of organics and radionuclides combined (Nriagu *et al.*, 1988, *Nature* 333:1340138). Since soil and water contamination results in the uptake of heavy metals and toxins by crop plants, and eventually humans, there remains a need for a means of manipulating the ability of a plant to sequester compounds from the soil in order to better manage soil detoxification through bioremediation using native species or genetically engineered organisms.

Regarding herbicides, these compounds are generally low molecular weight, lipophilic compounds that readily penetrate cells in a passive manner. Having entered cells, herbicides inhibit plant-specific processes such as photosynthetic electron transport (e.g., atrazine, chlortoluron) or the biosynthesis of essential amino acids (e.g., glyphosate, chlorsulfuron or phosphotricine), porphyrins (e.g., acidofluorfen), carotenoids (e.g., norflurazon), fatty acids (e.g., diclofop) or cellulose (e.g., dichlobenil) (Boger *et al.*, 1989, *Target Sites of Herbicide Action*, CRC Press, Boca Raton, FL; Devine *et al.*, 1993, *Physiology of Herbicide Action*, Prentice Hall, Englewood Cliffs, NJ). Plants that are naturally tolerant of certain herbicides either contain a cellular target that does not interact with the herbicide, have efficient systems for inactivation of the herbicide, or have a high capacity for excluding or eliminating the herbicide from the target.

Herbicide metabolism comprises the three phases described above for general xenobiotic metabolism. The first two phases (the first being oxidation and hydrolysis and the second being conjugation with GSH or glucose) contribute to detoxification by decreasing the intrinsic biochemical activity of the herbicide and/or by increasing its hydrophilicity. These two phases render the herbicide less mobile in the plant. The third phase (compartmentation) is often critical for sustained detoxification since the conjugates themselves may interfere with metabolism. For example, the herbicide synergist tridiphane, is converted to its corresponding GS-conjugate in plants to generate a potent inhibitor of atrazine metabolism. (Lamoureux *et al.*, 1986, *Pestic. Biochem. Physiol.* 26:323-342).

Likewise, and more generally, GS-conjugates of any given herbicide would be expected to act as end-product inhibitors of GSTs and thereby impair long-term detoxification unless they are removed from the intracellular compartment, usually the cytosol, in which they are formed. Since the vacuolar GS-X pump of plants is known to transport several GS-herbicide conjugates, for example, those of the chloroacetanilide herbicides (metolachlor) and triazines (simetryn) (Martinoia *et al.*, 1993, *supra*; Li *et al.*, 1995, *supra*), there is a long felt need for a knowledge of the

molecular identity of this transporter or family of transporters. Such knowledge will enable the development of new strategies for increasing or decreasing the resistance of plants to herbicides.

5 With regard to plant-pathogen interactions, a key event in the disease resistance response of legumes is the rapid and localized accumulation of isoflavonoid phytoalexins. The majority of the research on plant-pathogen interactions has centered on the enzymology and molecular biology of the isoflavonoid biosynthetic pathway (Dixon *et al.*, 1995, *Physiol. Plant* 93:385). However, the mechanism and sites of  
10 intracellular accumulation of these compounds is not understood. Since many isoflavonoid phytoalexins are as toxic to the host plant as they are to its pathogens, the discovery of the molecular mechanism by which these compounds are sequestered within a plant is crucial to the development of plants with increased pathogen resistance.

15 With regard to plant pigmentation, functional analyses of the maize gene, *Bronze-2*, which participates in anthocyanin pigment biosynthesis, suggest that one of the endogenous substrates for the plant vacuolar GS-X pump are anthocyanin-GS conjugates (Marrs *et al.*, 1995, *Nature*, 375:397-400). Anthocyanins share a common biosynthetic origin and core structure based on cyanidin-3-glucoside. It is through the species-specific decoration of cyanidin-3-glucoside by hydroxylation,  
20 methylation, glucosylation and acylation that the wide spectrum of red, blue and purple colors in the vacuoles of flowers, fruits and leaves is produced. The molecular nature of the plant GS-X pump which mediates transport of anthocyanin-GS conjugates was not known in the art until the present invention. There remains a need to determine the molecular nature of the GS-X pump responsible for transport of anthocyanin-GS  
25 conjugates in order that plant coloration may be manipulated at the molecular level.

The present invention satisfies the aforementioned needs.

## BRIEF SUMMARY OF THE INVENTION

The invention includes an isolated DNA encoding a plant GS-X pump polypeptide. In one aspect, the isolated DNA is selected from the group consisting of DNA comprising *AtMRP1* and *AtMRP2*, and any mutants, derivatives, homologs and fragments thereof encoding GS-X pump activity.

The invention also includes an isolated preparation of a polypeptide comprising a plant GS-X pump. In one aspect of this aspect of the invention, the polypeptide is selected from the group consisting of *AtMRP1*, *AtMRP2*, and any mutants, derivatives, homologs and fragments thereof having GS-X pump activity.

Also included in the invention is a recombinant cell comprising an isolated DNA encoding a plant GS-X pump polypeptide. In one aspect, the cell is selected from the group consisting of a prokaryotic cell and a eukaryotic cell.

Further included in the invention is a vector comprising an isolated DNA encoding a plant GS-X pump polypeptide.

The invention also includes an antibody specific for a plant GS-X pump polypeptide.

In addition, an isolated preparation of a nucleic acid which is in an antisense orientation to all or a portion of a plant GS-X pump gene is included in the invention and a cell and a vector comprising this isolated preparation of a nucleic acid are further included.

The invention also relates to a transgenic plant, the cells, seeds and progeny of which comprise an isolated DNA encoding a plant GS-X pump.

In addition, the invention relates to a transgenic plant, the cells, seeds and progeny of which comprise an isolated preparation of a nucleic acid which is in an antisense orientation to all or a portion of a plant GS-X pump gene.

Further, there is included a transgenic plant, the cells, seeds and progeny of which comprise an isolated DNA encoding YCF1, or any mutants, derivatives, homologs and fragments thereof having YCF1 activity.

The invention further relates to an isolated DNA comprising a plant GS-X pump promoter sequence. In one aspect, the promoter sequence is selected from the group consisting of an *AtMRP1* and an *AtMRP2* promoter sequence.

5 Also included in this aspect of the invention is a cell and a vector comprising an isolated DNA comprising a plant GS-X plant promoter sequence.

The invention additionally relates to a transgenic plant, the cells, seeds and progeny of which comprise a transgene comprising an isolated DNA comprising a GS-X pump promoter sequence, wherein the GS-X pump promoter sequence is selected from the group consisting of an *AtMRP1*, an *AtMRP2* and a *YCF1* promoter sequence.

10 The promoter sequence may also have operably fused thereto a reporter gene.

There is also included in the invention a method of identifying a compound capable of affecting the expression of a plant GS-X gene. The method comprises providing a cell comprising an isolated DNA comprising a plant GS-X pump promoter sequence having a reporter sequence operably linked thereto, adding to the

15 cell a test compound, and measuring the level of reporter gene activity in the cell, wherein a higher or a lower level of reporter gene activity in the cell compared with the level of reporter gene activity in a cell to which the test compound was not added, is an indication that the test compound is capable of affecting the expression of a plant GS-X pump gene.

20 In addition, the invention relates to a method of removing xenobiotic toxins from soil. The method comprises growing in the soil a transgenic plant of comprising an isolated DNA encoding a GS-X pump.

Also included is a method of removing heavy metals from soil comprising growing in the soil a transgenic plant of comprising an isolated DNA

25 encoding a GS-X pump.

The invention further relates to a method of generating a transgenic pathogen resistant plant comprising introducing to the cells of the plant an isolated DNA encoding a GS-X pump, wherein the pump is capable of transporting glutathionated isoflavonoid alexins into the cells of the plant.

Additionally, there is included a method of manipulating plant pigmentation comprising modulating the expression of a GS-X pump protein in the plant, wherein the GS-X pump protein is selected from the group consisting of AtMRP1, AtMRP2 and YCF1.

5           The invention also relates to a method of alleviating oxidative stress in a plant comprising introducing into the cells of the plant DNA encoding a GS-X pump, wherein the DNA is selected from the group consisting of DNA encoding AtMRP1, AtMRP2 and YCF1.

10           Further included is a method of manipulating the expression of a gene in a plant cell. The method comprises operably fusing a GS-X pump promoter sequence to the DNA sequence encoding the gene to form a chimeric DNA, and generating a transgenic plant, the cells of which comprise the chimeric DNA, wherein upon activation of the GS-X pump promoter sequence, the expression of the gene is manipulated.

## 15           BRIEF DESCRIPTION OF THE DRAWINGS

          Figure 1 is a series of graphs depicting differential sensitivities of DYT165 cells (wild type, Figure 1A) and DTY167 cells (*ycf1Δ* mutant, Figure 1B) to growth inhibition by 1-chloro-2,4-dinitrobenzene (CDNB). Cells were grown at 30°C for 24 hours to an OD<sub>600 nm</sub> of approximately 1.4 in YPD medium before inoculation of aliquots into 15 ml volumes of the same medium containing 0-60 μM CDNB. OD<sub>600 nm</sub> was measured at the times indicated.

20           Figure 2 is a graph depicting the time course of [<sup>3</sup>H]DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells. Uptake was measured in the absence (-MgATP) or presence of 3 mM MgATP (+MgATP) in reaction media containing 66.2 μM [<sup>3</sup>H]DNP-GS, 10 mM creatine phosphate, 16 units/ml creatine kinase, 50 mM KCl, 0.1% (w/v) bovine serum albumin, 400 mM sorbitol, and 25 mM Tris-MES (pH 8.0) at 25°C. Values shown are means ± S.E. (*n* = 3).

Figure 3 is a series of graphs depicting the kinetics of uncoupler-insensitive [ $^3\text{H}$ ]DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells. Figure 3A: MgATP concentration-dependence of uncoupler-insensitive uptake. Figure 3B: DNP-GS concentration-dependence of MgATP-dependent uncoupler-insensitive uptake. The MgATP concentration-dependence of uptake was measured with 66.2  $\mu\text{M}$  [ $^3\text{H}$ ] DNP-GS. The DNP-GS concentration-dependence of uptake was measured with 3 mM MgATP. Uptake was allowed to proceed for 10 minutes in standard uptake medium containing 5  $\mu\text{M}$  gramicidin D. The kinetic parameters for vacuolar membrane vesicles purified from DTY165 cells were  $K_{\text{m(MgATP)}}$   $86.5 \pm 29.5 \mu\text{M}$ ,  $K_{\text{m(DNP-GS)}}$   $14.1 \pm 7.4 \mu\text{M}$ ,  $V_{\text{max(MgATP)}}$   $38.4 \pm 5.6 \text{ nmol/mg/10 minutes}$ ,  $V_{\text{max(DNP-GS)}}$   $51.0 \pm 6.3 \text{ nmol/mg/10 minutes}$ . The lines of best fit and kinetic parameters were computed by nonlinear least squares analysis (Marquardt, 1963, *J. Soc. Ind. Appl. Math.* 11:431-441). Values shown are means  $\pm$  S.E. ( $n = 3$ ).

Figure 4 is a series of graphs depicting sucrose density gradient fractionation of vacuolar membrane-enriched vesicles prepared from DYT165 cells. One ml (1.1 mg protein) of partially purified vacuolar membrane vesicles derived from vacuoles prepared by the Ficoll flotation technique were applied to a linear sucrose density gradient (10-40%, w/v) and analyzed for protein (Figure 4A),  $\alpha$ -mannosidase activity (Figure 4B), V-ATPase activity (Figure 4C), and MgATP-dependent, uncoupler-insensitive [ $^3\text{H}$ ]DNP-GS uptake (Figure 4D). [ $^3\text{H}$ ]DNP-GS uptake and enzyme activity were assayed as described herein in Table 4 and the accompanying text.

Figure 5 includes a graph (Figure 5A) depicting the effect of transformation with pYCF1-HA or pRS424 on MgATP-dependent, uncoupler-insensitive [ $^3\text{H}$ ]DNP-GS uptake by vacuolar membranes purified from DTY165 and DTY167 cells. Uptake was measured in standard uptake medium containing 66.2  $\mu\text{M}$  [ $^3\text{H}$ ]DNP-GS and 5  $\mu\text{M}$  gramicidin D. Also shown (Figure 5B) is an image of a gel depicting immunoreaction of vacuolar membrane proteins prepared from pYCF1-HA-

transformed and pRS424-transformed DTY165 and DTY167 cells with mouse monoclonal antibody raised against the 12CA5 epitope of human influenza hemagglutinin. All lanes were loaded with 25  $\mu$ g of delipidated membrane protein and subjected to SDS-polyacrylamide gel electrophoresis and Western analysis as described  
5 herein. The  $M_r$  of YCF1-HA (boldface type) and the positions of the  $M_r$  standards are indicated on the figure.

Figure 6 is a series of graphs depicting transformation with pYCF1-HA (Figure 6A) or pRS424 (Figure 6B) on the sensitivity of DTY167 cells to growth retardation by CDNB. Cells were grown at 30°C for 24 hours to an  $OD_{600\text{ nm}}$  of  
10 approximately 1.4 in AHC medium (Kim *et al.*, 1994, *Proc. Natl. Acad. Sci. USA* 91:6128-6132) before inoculation of aliquots into 15 ml volumes of the same medium containing 0-60  $\mu$ M CDNB.  $OD_{600\text{ nm}}$  was measured at the times indicated.

Figure 7 is a series of photomicrographs of DTY165 (Figures 7A and 7C) and DTY167 cells (Figures 7B and 7D) after incubation with monochlorobimane.  
15 Cells were grown in YPD medium for 24 hours at 30°C and 100  $\mu$ l aliquots of cell suspensions were transferred into 15 ml volumes of fresh YPD medium containing 100  $\mu$ M monochlorobimane. After incubation for 6 hours, the cells were washed and examined in fluorescence (Figures 7C and 7D) or Nomarski mode (Figures 7A and 7B) as described herein.

Figure 8 is a series of graphs depicting uptake of  $Cd^{2+}$  into vacuolar  
20 membrane vesicles purified from DTY165 and DTY167 cells. Uptake of  $^{109}Cd^{2+}$  by DTY165 membranes (Figure 8A) or DTY167 membranes (Figure 8B) was measured in the absence of MgATP plus (O) or minus GSH (1 mM) ( $\square$ ) or in the presence of MgATP (3 mM) plus ( $\bullet$ ) or minus ( $\blacksquare$ ) GSH.  $^{109}Cd_2SO_4$  and gramicidin-D were  
25 added at concentrations of 80  $\mu$ M and 5  $\mu$ M, respectively. Figure 8C: Rate of  $^{109}Cd^{2+}$  uptake by DTY165 membranes plotted as a function of the total concentration of  $Cd^{2+}$  ( $[Cd^{2+}]_{total}$ ) added to uptake medium containing 1 mM GSH, 3 mM MgATP and 5  $\mu$ M gramicidin-D. Values shown are means  $\pm$  SE ( $n = 3-6$ ).



Figure 9 is a series of graphs depicting purification of cadmium glutathione complexes by gel-filtration (Figure 9A) and anion-exchange chromatography (Figures 9B and 9C). Twenty mM  $^{109}\text{Cd}_2\text{SO}_4$  was incubated with 40 mM GSH at 45°C for 24 hours and the mixture was chromatographed on Sephadex G-15 to resolve a high molecular weight  $^{109}\text{Cd}$ -labeled component ( $HMW$ - $^{109}\text{Cd.GS}$ ) from a low molecular weight component ( $LMW$ - $^{109}\text{Cd.GS}$ ) (Figure 9A). The peaks corresponding to  $HMW$ - $^{109}\text{Cd.GS}$  and  $LMW$ - $^{109}\text{Cd.GS}$  were then chromatographed on Mono-Q and eluted with a linear NaCl gradient (---) (Figure 9B and 9C).  $^{109}\text{Cd}$  (cpm  $\times 10^{-3}$ ) was determined on 5  $\mu\text{l}$  aliquots of the column fractions by liquid scintillation counting.

Figure 10 is a series of graphs depicting the kinetics of MgATP-dependent, uncoupler-insensitive  $^{109}\text{Cd.GS}_2$  ( $HMW$ - $^{109}\text{Cd.GS}$ , Figure 10A) and  $^{109}\text{Cd.GS}$  ( $LMW$ - $^{109}\text{Cd.GS}$ , Figure 10B) uptake. DNP-GS was added at the concentrations ( $\mu\text{M}$ ) indicated to DTY165 membranes ( $\bullet, \circ, \blacksquare, \square, \Delta$ ) or DTY167 membranes ( $\diamond$ ). A secondary plot of the apparent Michaelis constants for  $\text{Cd.GS}_2$  uptake ( $K_m^{\text{app}}/\text{Cd.GS}_2$ ) as a function of DNP-GS concentration is shown (Figure 10C). The kinetic parameters for  $\text{Cd.GS}_2$  transport by DTY165 membranes were  $K_m$ ,  $39.1 \pm 14.1 \mu\text{M}$ ,  $V_{\text{max}}$ ,  $157.2 \pm 30.4 \text{ nmol/mg/10 minutes}$  and  $K_{i(\text{DNP-GS})}$ ,  $11.3 \pm 2.1 \mu\text{M}$ . Kinetic parameters were computed by nonlinear least squares analysis (Marquardt, 1963, *supra*). Values shown are means  $\pm$  SE ( $n = 6$ ).

Figure 11 is a graph depicting matrix-assisted laser desorption mass spectrometry (MALD-MS) of  $HMW$ - $\text{Cd.GS}$ . MALD-MS was performed on Sephadex G-15-, Mono-Q-purified  $HMW$ - $\text{Cd.GS}$  as described herein. The molecular structure inferred from a mean  $m/z$  ratio of  $725.4 \pm 0.7$  ( $n = 9$ ) and average  $\text{Cd.GS}$  stoichiometry of 0.5 [*bis*(glutathionato)cadmium,  $\text{Cd.GS}_2$ , molecular weight 724.6 Da] is shown.

Figure 12 is an image of a gel depicting induction of *YCF1* expression and *YCF1*-dependent  $\text{Cd.GS}_2$  and DNP-GS transport by pretreatment of DTY165 cells with  $\text{CdSO}_4$  ( $\text{Cd}^{2+}$ , 200  $\mu\text{M}$ ) or CDNB (150  $\mu\text{M}$ ) for 24 hours. *YCF1*-specific mRNA and 18S rRNA were detected in the total RNA extracted from control or pretreated

cells (10 µg/lane) by RNase protection. Uptake of  $^{109}\text{Cd.GS}_2$  (50 µM) or  $[^3\text{H}]\text{DNP-GS}$  (66.2 µM) by vacuolar membrane vesicles was measured in standard uptake medium containing 5 µM gramicidin-D. Values shown are means  $\pm$  SE ( $n = 3$ ).

5                      Figure 13A and 13B is the sequence of *AtMRP2* cDNA (SEQ ID NO:1). Lower case letters correspond to 5'- and 3'-untranslated regions (UTRs).

                    Figure 14A-D is the genomic sequence of *AtMRP2* (SEQ ID NO:2). Lower case letters at the beginning and end of the sequence correspond to 5'- and 3'-UTRs, respectively; lower case letters nested within the sequence correspond to introns.

10                     Figure 15 is the deduced amino acid sequence of *AtMRP2* (SEQ ID NO:3).

                    Figure 16A and 16B is the sequence of *AtMRP1* cDNA (SEQ ID NO:4). Lower case letters correspond to 5'- and 3'-UTRs.

15                     Figure 17A-D is the genomic sequence of *AtMRP1* (SEQ ID NO:5). Lower case letters at the beginning and end of the sequence correspond to 5'- and 3'-UTRs, respectively; lower case letters nested within the sequence correspond to introns.

                    Figure 18 is the deduced amino acid sequence of *AtMRP1* (SEQ ID NO:6).

20                     Figure 19 is a series of graphs depicting the time course and concentration-dependence of DNP-GS uptake in *AtMRP1*-transformed yeast. Figure 19A is a graph depicting the time course of  $[^3\text{H}]\text{DNP-GS}$  uptake by membrane vesicles purified from pYES3-*AtMRP1*-transformed or pYES3-transformed DTY168 cells. MgATP-dependent uptake was measured in reaction media containing 61.3 µM  $[^3\text{H}]\text{DNP-GS}$ , 5 µM gramicidin-D, 10 mM creatine phosphate, 16 units/ml creatine kinase, 50 mM KCl, 1 mg/ml bovine serum albumin, 400 mM sorbitol and 25 mM Tris-Mes (pH 8.0) at 25°C. Values shown are means  $\pm$  SE ( $n = 3$ ). Figure 19B is a graph depicting concentration dependence of MgATP-dependent, uncoupler-insensitive uptake of  $[^3\text{H}]\text{DNP-GS}$  by membrane vesicles purified from pYES3-*AtMRP1*-

25

transformed DTY168 cells. Uptake was allowed to proceed for 10 minutes in standard uptake medium containing 5  $\mu$ M gramicidin-D. The kinetic parameters for uptake were  $K_{m(\text{DNP-GS})}$   $49.7 \pm 15.4 \mu\text{M}$ ,  $V_{\text{max}}$   $6.0 \pm 1.7 \text{ nmol/mg/10 minutes}$ . The lines of best fit and kinetic parameters were computed by nonlinear least squares analysis (Marquardt, 1963, supra). Values shown are means  $\pm$  SE ( $n = 3$ ).

Figure 20 is a series of graphs depicting sensitivity of MgATP-dependent, uncoupler-insensitive [ $^3\text{H}$ ]DNP-GS uptake by membrane vesicles purified from pYES3-*AtMRP1*-transformed and pYES3-transformed DTY168 cells. Uptake was measured for 10 minutes in standard uptake medium containing the indicated concentrations of vanadate. In Figure 20A, there is a graph depicting total MgATP-dependent, uncoupler-insensitive [ $^3\text{H}$ ]DNP-GS uptake by membrane vesicles purified from pYES3-*AtMRP1*-transformed and pYES-transformed DTY168 cells. In Figure 20B, there is a graph depicting *AtMRP1*-dependent uptake.  $I_{50}$  (exclusive of uninhibitable *AtMRP1*-independent component) =  $8.3 \pm 3.2 \mu\text{M}$ . Values shown are means  $\pm$  SE ( $n = 3$ ).

Figure 21 is a series of graphs depicting the hydropathy alignment of *AtMRP2*, *AtMRP1*, *S. cerevisiae* YCF1 (*ScYCF1*), human *MRP1* (*HmMRP1*) and rat *cMOAT* (*RtCMOAT*).

Figure 22 is a diagram depicting domain comparisons between *AtMRP1*, *ScYCF1*, *HmMRP1*, *RtCMOAT*, rabbit EBCR (*RbEBCR*) and *HmCFTR*. The domains indicated are the N-terminal extension ( $\text{NH}_2$ ), first and second sets of transmembrane spans (TM1 and TM2, respectively), first and second nucleotide binding folds (NBF1 and NBF2, respectively), putative CFTR-like regulatory domain (R), and the C-terminus (COOH).

Figure 23 is the promoter sequence of the *Arabidopsis AtMRP1* gene (SEQ ID NO:7). Discrete elements which are present in the promoter sequence are indicated in boldface letters.

Figure 24 is the promoter sequence of the *Arabidopsis AtMRP2* gene (SEQ ID NO:8). Discrete elements which are present in the promoter sequence are indicated in boldface letters.

5 Figure 25 is a graph depicting MgATP-dependence of [ $^3$ H]medicarpin uptake by vacuolar membrane vesicles before (Medicarpin/GSH) and after maize GST-mediated conjugation with GSH (Medicarpin-GS). [ $^3$ H]medicarpin or [ $^3$ H]medicarpin-GS was added at a concentration of 65  $\mu$ M. MgATP was either omitted (-MgATP) or added at a concentration of 3 mM (+MgATP). Values shown are means  $\pm$  SE (n = 3).

10 Figure 26 is a graph depicting concentration dependence of MgATP-dependent, uncoupler-insensitive [ $^3$ H]medicarpin-GS uptake into vacuolar membrane vesicles. Uptake was allowed to proceed for 20 minutes in standard uptake medium containing 3 mM MgATP and 5  $\mu$ M gramicidin D. The kinetic parameters were  $K_m$  21.5  $\pm$  15.5  $\mu$ M and  $V_{max}$  77.8  $\pm$  23.3 nmol/mg/20 minutes. Values shown are means  $\pm$  SE (n = 3).

15 Figure 27 is a series of graphs depicting concentration-dependence of MgATP-dependent, uncoupler-insensitive C<sub>3</sub>G-GS, IAA-GS and ABA-GS uptake by vacuolar membrane vesicles purified from *V. radiata* (Figure 27A) and *Z. mays* (Figure 27B). Uptake was allowed to proceed for 10 minutes in reaction medium containing 50  $\mu$ M GS-conjugate, 400 mM sorbitol, 3 mM MgATP, 50 mM KCl, 0.1% (w/v) BSA, 5  $\mu$ M gramicidin-D and 25 mM Tris-Mes (pH 8.0) at 25°C. Values shown  
20 are means  $\pm$  SE (n = 3).

Figure 28 is an image of a photograph depicting the growth of wild type (WT) and *YCF1* transgenic *Arabidopsis* (*YCF1*) seeds on media containing CdSO<sub>4</sub> (200  $\mu$ M) or 1-chloro-2,4-dinitrobenzene (CDNB, 25  $\mu$ M). Transgenic plants were  
25 generated as described herein.

## DETAILED DESCRIPTION OF THE INVENTION

The invention is based upon the molecular identification of a new class of membrane transporter in yeast and plants, the GS-X pump. As a result of the present

invention, new insights into the membrane transport phenomena associated with heavy metal tolerance, herbicide detoxification, plant-pathogen interactions, plant responses to (phyto)hormones, plant pigmentation and bioremediation are evident. These insights provide a means, as is evident from the description of the present invention, for the manipulation of plants and the cells thereof, to affect heavy metal tolerance, herbicide detoxification, plant-pathogen interactions, plant responses to (phyto)hormones, plant pigmentation and bioremediation.

The process of "storage excretion" is a necessity for plants. Whereas mammals have the option of excreting GS-conjugates to the extracellular medium for elimination by the kidneys, plants are nearly totally reliant on the sequestration of noxious compounds in the central vacuole, which frequently accounts for 40-90% of total intracellular volume. Due to the virtual absence of specialized excretory organs and the presence of massive vacuoles in plants, a process (intracellular compartmentation) that is probably only an intermediate step in the elimination of xenobiotics from the cytosol of mammalian cells, is believed to constitute a terminal phase of detoxification in plants.

The data which are described herein establish that the yeast gene *YCF1* and two plant homologs of *YCF1*, *AtMRP1* and *AtMRP2*, isolated from *Arabidopsis thaliana*, each encode a vacuolar GS-X pump. The data further establish that the GS-X pump participates in herbicide metabolism (exemplified by organic xenobiotic transport), heavy metal sequestration (exemplified by cadmium transport), plant-pathogen interactions (exemplified by vacuolar uptake of medicarpin), plant cell pigmentation (exemplified by transport of glutathionated anthocyanins) and plant hormone metabolism (exemplified by the transport of glutathionated auxins).

The plant *AtMRP1* and *AtMRP2* gene products use MgATP as an energy source for the transport of glutathionated derivatives of both endogenous and exogenous compounds in plants and thus, the discovery of these genes in the present invention is important at three levels. The identification of these genes and their encoded products represents the first identification of ABC transporters in plants for

which a biochemical function is defined. The discovery establishes, contrary to the prevailing chemiosmotic model for solute transport in plants, that many energy-dependent solute transport processes in plants are not driven by a transmembrane H<sup>+</sup> electrochemical potential difference. Further, the identification and isolation of these genes and their encoded products permits a plant element, critical for removal of compounds from the cytosol that can form glutathionine S-conjugates, to be manipulated.

It has been discovered in the present invention that two plant genes, *AtMRP1* and *AtMRP2*, are the structural and functional homologs of the gene encoding yeast YCF1. Proteins encoded by plant *AtMRP1* and *AtMRP2* thus represent a new subclass of ATP binding cassette transporters.

It has been further discovered in the present invention that the yeast YCF1 protein, a GS-X pump, is capable of MgATP-energized transport of organic GS-conjugates and of MgATP-energized transport of cadmium upon complexation with GSH. In addition, when plants have introduced into the cells thereof the *YCF1* gene (a transgenic plant comprising *YCF1*), expression of *YCF1* therein confers upon the plants resistance to both inorganic and organic xenobiotics exemplified by cadmium and 1-chloro-2,4-dinitrobenzene, respectively.

Also discovered in the present invention is the fact that *AtMRP1* and *AtMRP2*, when expressed in a strain of yeast which is deficient in YCF1, can substitute for *YCF1* as a GS-X pump. In addition, transformation of plants by *YCF1* confers upon the plant properties which are characteristic of *YCF1* gene expression. Thus, it appears that *YCF1* and the *AtMRP* genes are essentially functionally interchangeable.

In addition, there is provided as part of the invention the promoter/regulatory sequences which control expression of the plant *AtMRP1* and *AtMRP2* genes of the invention. These promoter sequences are useful for the identification of compounds which affect expression of these genes in plants and for conferring on other genes the ability to respond to factors that modulate *AtMRP1* and/or *AtMRP2* expression.

Further discovered in the present invention is the fact that the plant GS-  
X pump serves to facilitate the vacuolar storage of antimicrobial compounds induced  
following the hypersensitive response to fungal pathogens in the healthy cells  
surrounding fungally-induced lesions. Such a process is believed to limit the spread of  
5 tissue damage by limiting propagation of the pathogen and spatially delimiting the  
toxic action of the phytoalexin itself.

Ascription of specific enzymic and regulatory roles to most of the genes  
of the anthocyanin biosynthetic pathway has been achieved by genetic and biochemical  
studies of maize with one notable exception, the *Bronze-2* gene. It is known that the  
10 characteristic coloration of *Bronze-2* (*bz2*) mutants is a consequence of the  
accumulation of cyanidin-3-glucoside in the cytosol. However, in wild type (*Bz2*)  
plants, anthocyanins are transported into the vacuole and become purple or red. In the  
mutant (*bz2*) plants, anthocyanin is restricted to the cytoplasm where it is oxidized to a  
brown ("bronze") pigment. The biochemical basis for the accumulation of  
15 anthocyanins in the cytosol is not known. However, Marrs *et al.*, (1995, *supra*) have  
discovered that *Bz2* encodes a glutathione S-transferase which is responsible for  
conjugating anthocyanin with GSH. It has now been discovered in the present  
invention that the plant GS-X pump is the entity responsible for the delivery of  
glutathionated anthocyanins into the vacuole.

20 Identification of the GS-X pump at the molecular level has served to  
confirm its wide distribution and demonstrate that these transporters constitute a  
multigene family within the ABC transporter superfamily. The critical finding was that  
overexpression of the human multidrug resistance-associated protein (*MRP1*) gene  
(Cole *et al.*, 1992, *supra*) confers increased MgATP-dependent GS-conjugate transport  
25 (Muller *et al.*, 1994 *supra*; Leier *et al.*, 1994, J. Biol. Chem. 269:27807-27810).

Several other closely related GS-X pump genes have been characterized.  
For example, a liver-specific GS-X pump (*cMOAT*), mutation of which is believed to  
cause hereditary hyperbilirubinemia, has been cloned (Paulusma *et al.*, 1996, Science  
271:1126-1128). The present invention establishes that YCF1 is a GS-X pump. In

addition, as will become apparent from a reading of the present description, two plant genes, *AtMRP1* and *AtMRP2* have been discovered in the present invention to encode homologs of MRP1, YCF1 and cMOAT.

5 The identification of YCF1 as a vacuolar GS-X pump is described in detail in the experimental details section. Similarly, the identification of two plant homologs of YCF1, *AtMRP1* and *AtMRP2*, is also described in detail in the experimental details section. Once armed with the present invention, the skilled artisan will know how to identify and isolate genes encoding other plant GS-X pumps involved in sequestration of a variety of compounds in plants by following the procedures  
10 described herein.

A plant gene encoding a GS-X pump is isolated using any one of several known molecular procedures. For example, primers comprising conserved regions of the sequences of any of *YCF1*, *AtMRP1* or *AtMRP2*, or in fact primers comprising conserved regions of any MRP subclass (*i.e.*, probes directed to human *MRP1 cMOAT*,  
15 and other MRP genes) may be used as probes to isolate, by polymerase chain reaction (PCR) or by direct hybridization, as yet unknown *YCF1*, *AtMRP1* or *AtMRP2* homologs in a DNA library comprising specific plant DNAs. Alternatively, antibodies directed against YCF1, *AtMRP1* or *AtMRP2* may be used to isolate clones encoding a GS-X pump from an expression library comprising specific plant DNAs. The isolation  
20 of primers, probes, molecular cloning and the generation of antibodies are procedures that are well known in the art and are described, for example, in Sambrook *et al.* (1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York) and in Harlow *et al.* (1988, *Antibodies, A Laboratory Manual*, Cold Spring Harbor, New York).

25 The invention includes an isolated DNA encoding a plant GS-X pump capable of transporting a glutathionated compound across a biological membrane. Preferably, the membrane is derived from a cell. Preferably, the DNA encoding a plant GS-X pump is at least about 40% homologous to at least one of *YCF1*, *AtMRP1* or *AtMRP2*. More preferably, the isolated DNA encoding a plant GS-X pump is at least



about 50%, even more preferably, at least about 60%, yet more preferably, at least about 70%, even more preferably, at least about 80%, yet more preferably, at least about 90% homologous, and more preferably, at least about 99% homologous to at least one of *YCF1*, *AtMRP1* or *AtMRP2*. More preferably, the isolated DNA encoding a plant GS-X pump is *Arabidopsis AtMRP1* or *AtMRP2*. Most preferably, the isolated DNA encoding a plant GS-X pump is SEQ ID NOS:1, 2, 4 or 5.

Thus, the invention should be construed to include genes which encode *Arabidopsis AtMRP1* and *AtMRP2* and *Arabidopsis AtMRP1* and *AtMRP2*-related genes.

By "GS-X pump" as used herein, is meant a protein which transports a glutathione-conjugated compound across a biological membrane.

By the term "DNA encoding a GS-X pump" as used herein is meant a gene encoding a polypeptide capable of transporting a glutathionated compound across a biological membrane.

By "*AtMRP*-related gene" as used herein, is meant a gene encoding a GS-X pump which is a member of the *MRP/YCF1/cMOAT* family of genes. An *AtMRP1* or *AtMRP2*-related gene may be present in a cell which also encodes an *AtMRP* gene or it may be present in a different cell and in a different plant species.

As described in the Experimental Detail section, *AtMRP* genes encode proteins which have specific domains located therein, namely, the N-terminal extension, transmembrane spans, TM1 and TM2, nucleotide binding folds, NBF1 and NBF2, putative CFTR-like regulatory domain (R) and the C-terminus. An *AtMRP*-related gene is therefore also one in which selected domains in the related protein share significant homology (at least about 40% homology) with the same domains in either of *YCF1*, *AtMRP1* or *AtMRP2*. For example, when the R-domain in the *AtMRP*-related protein shares at least about 40% homology with the R domain in *YCF1*, *AtMRP1* or *AtMRP2*, and when the product of that is a GS-X pump, then that gene is an *AtMRP*-related gene. Similarly, when the N-terminal extension in the *AtMRP*-related protein shares at least about 40% homology with the N-terminal extension in

YCF1, AtMRP1 or AtMRP2, and when the product of that is a GS-X pump, then that gene is an *AtMRP*-related gene. It will be appreciated that the definition of an *AtMRP*-related gene encompasses those genes having at least about 40% homology in any of the described domains contained therein with the same or a similar domain in either of YCF1, AtMRP1 or AtMRP2. In addition, when the term homology is used herein to refer to the domains of these proteins, it should be construed to be applied to homology at both the nucleic acid and the amino acid levels.

While a significant homology between similar domains in *AtMRP*-related genes or their protein products is considered to be at least about 40%, preferably, the homology between domains is at least about 50%, more preferably, at least about 60%, even more preferably, at least about 70%, even more preferably, at least about 80%, yet more preferably, at least about 90% and most preferably, the homology between similar domains is about 99% between a domain in an *AtMRP*-related gene or protein product thereof, and at least one of YCF1, *AtMRP1* or *AtMRP2* or the protein products thereof.

Plants from which *AtMRP1*, *AtMRP2* or YCF1 related genes may be isolated include any plant in which the GS-X pump is found, including, but not limited to, soybean, castor bean, maize, petunia, potato, tomato, sugar beet, tobacco, oats, wheat, barley, pea, faba bean and alfalfa.

By the term "glutathionated-conjugated compound" as used herein is meant a compound, *e.g.*, a metal, a xenobiotic, a isoflavonoid phytoalexin, anthocyanin or auxin, which is chemically conjugated to glutathione. Conjugation of compounds to glutathione occurs naturally within cells and organisms and may also be accomplished enzymatically or non-enzymatically *in vitro* as described herein in the experimental details section.

Also included in the invention is an isolated DNA encoding a biologically active polypeptide fragment of a plant GS-X pump. Preferably, the isolated DNA encoding a biologically active polypeptide fragment of a plant GS-X pump is at least about 40% homologous to a biologically active polypeptide fragment

of at least one of YCF1, AtMRP1 or AtMRP2. More preferably, the isolated DNA encoding a biologically active polypeptide fragment of a plant GS-X pump is at least about 50%, even more preferably, at least about 60%, yet more preferably, at least about 70%, even more preferably, at least about 80%, yet more preferably, at least about 90%, and even more preferably, at least about 99% homologous to a biologically active polypeptide fragment of at least one of YCF1, AtMRP1 or AtMRP2. Most preferably, the isolated DNA encoding a biologically active polypeptide fragment of a plant GS-X pump is a biologically active polypeptide fragment of *Arabidopsis* AtMRP1 or AtMRP2.

Preferably, the isolated DNA encoding a biologically active polypeptide fragment of a plant GS-X pump is about 200 nucleotides in length. More preferably, the isolated DNA encoding a biologically active polypeptide fragment of a plant GS-X pump is about 400 nucleotides, even more preferably, at least about 600, yet more preferably, at least about 800, even more preferably, at least about 1000, and more preferably, at least about 1200 nucleotides in length.

The invention further includes a vector comprising a gene encoding a plant GS-X pump and a vector comprising nucleic acid sequence encoding a biologically active fragment thereof. The procedures for the generation of a vector encoding a plant GS-X pump, or fragment thereof, are well known in the art once the sequence of the gene is known, and are described, for example, in Sambrook *et al.* (*supra*). Suitable vectors include, but are not limited to, disarmed *Agrobacterium* tumor-inducing (Ti) plasmids (e.g., pBIN19) containing the target gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Lagrimini *et al.*, 1990, Plant Cell 2:7-18) or its endogenous promoter (Bevan, 1984, Nucl. Acids Res. 12:8711-8721).

Also included in the invention is a cell comprising an isolated DNA encoding a plant GS-X pump and a cell comprising an isolated DNA encoding a biologically active fragment thereof. Such a cell is referred to herein as a "recombinant cell."

The procedures for the generation of a cell encoding a plant GS-X pump or fragment thereof, are well known in the art once the sequence of the gene is known, and are described, for example, in Sambrook *et al.* (*supra*). Suitable cells include, but are not limited to, yeast cells, bacterial cells, mammalian cells, and baculovirus-  
5 infected insect cells transformed with the gene for the express purpose of generating GS-X polypeptide. In addition, plant cells transformed with the gene for the purpose of producing cells and regenerated plants having increased resistance to and increased capacity for heavy metal accumulation, increased resistance to organic xenobiotics and increased capacity for organic xenobiotic accumulation or altered coloration.

10 The invention also includes an isolated preparation of a polypeptide comprising a plant GS-X pump capable of transporting a glutathionated compound across a biological membrane. Preferably, the isolated preparation of a polypeptide comprising a plant GS-X pump is at least about 30% homologous to at least one of YCF1, AtMRP1 or AtMRP2. More preferably, the isolated preparation of a  
15 polypeptide comprising a plant GS-X pump is at least about 40%, even more preferably, at least about 50%, yet more preferably, at least about 60%, even more preferably, at least about 70%, more preferably, at least about 80%, even more preferably, at least about 90% and more preferably, at least about 99% homologous to at least one of YCF1, AtMRP1 or AtMRP2. More preferably, the isolated preparation  
20 of a polypeptide comprising a plant GS-X pump is *Arabidopsis* AtMRP1 or AtMRP2. Most preferably, the isolated preparation of a polypeptide comprising a plant GS-X pump is SEQ ID NOS: 3 or 6.

Also included in the invention is an isolated preparation of a biologically active polypeptide fragment of a plant GS-X pump. Preferably, the  
25 isolated preparation of a biologically active polypeptide fragment of a plant GS-X pump is at least about 30% homologous to a biologically active polypeptide fragment of at least one of YCF1, AtMRP1 or AtMRP2. More preferably, the isolated preparation of a biologically active polypeptide fragment of a plant GS-X pump is at least about 40%, even more preferably, at least about 50%, yet more preferably, at least

about 60%, even more preferably, at least about 70% and yet more preferably, at least about 80%, even more preferably, at least about 90% and more preferably, at least about 99% homologous to a biologically active polypeptide fragment of at least one of YCF1, AtMRP1 or AtMRP2. Most preferably, the isolated preparation of a  
5 biologically active polypeptide fragment of a plant GS-X pump is a biologically active polypeptide fragment of *Arabidopsis* AtMRP1 or AtMRP2.

Preferably, the polypeptide in the isolated preparation of a biologically active polypeptide fragment of a plant GS-X pump is about 60 amino acids in length. More preferably, the polypeptide in the isolated preparation of a biologically active  
10 polypeptide fragment of a plant GS-X pump is about 130 amino acids, even more preferably, at least about 200, yet more preferably, at least about 300, even more preferably, at least about 350, and more preferably, at least about 400 amino acids in length.

As used herein, the term "homologous" refers to the subunit sequence similarity between two polymeric molecules *e.g.*, between two nucleic acid molecules,  
15 *e.g.*, between two DNA molecules, or two polypeptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit, *e.g.*, if a position in each of two polypeptide molecules is occupied by phenylalanine, then they are homologous at that position. The homology between two sequences is a direct  
20 function of the number of matching or homologous positions, *e.g.*, if half (*e.g.*, 5 positions in a polymer 10 subunits in length) of the positions in two polypeptide sequences are homologous then the two sequences are 50% homologous; if 70% of the positions, *e.g.*, 7 out of 10, are matched or homologous, the two sequences share 70%  
25 homology. By way of example, the polypeptide sequences ACDEFG and ACDHIK (SEQ ID NOS:9 and 10, respectively) share 50% homology and the nucleotide sequences CAATCG and CAAGAC share 50% homology.

An "isolated DNA," as used herein, refers to a DNA sequence which has been separated from the sequences which flank it in a naturally occurring state, *e.g.*, a DNA fragment which has been removed from the sequences which are normally

adjacent to the fragment, *e.g.*, the sequences adjacent to the fragment in a genome in which it naturally occurs. The term also applies to nucleic acids which have been substantially purified from other components which naturally accompany the nucleic acid (*e.g.*, RNA, DNA or protein) in its natural state. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (*e.g.*, as a cDNA or a genomic or cDNA fragment produced by PCR or restriction enzyme digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

As used herein, the term "isolated preparation of a polypeptide" describes a polypeptide which has been separated from components which naturally accompany it. Typically, a polypeptide is isolated when at least 10%, more preferably at least 20%, more preferably at least 50%, more preferably at least 60%, even more preferably at least 75%, more preferably at least 90%, and most preferably at least 99% of the total material (by volume, by wet or dry weight, or by mole per cent or mole fraction) of a sample is the polypeptide of interest. The degree of isolation of the polypeptide can be measured by any appropriate method, *e.g.*, by column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis. For example, a polypeptide is isolated when it is essentially free of naturally associated components or when it is separated from the native compounds which accompany it in its natural state.

As used herein, by the term "biologically active" as it refers to GS-X pump activity as used herein, is meant a polypeptide, or a fragment thereof, which is capable of transporting a glutathionated compound across a biological membrane.

In summary, the invention should be construed to include DNA comprising *AtMRP1* and *AtMRP2*, and any mutants, derivatives, homologs and fragments thereof, which encode GS-X pump biological activity.

The invention further features an isolated preparation of a nucleic acid which is antisense in orientation to a portion or all of a plant GS-X pump gene, wherein the nucleic acid is capable of inhibiting expression of the GS-X pump gene when introduced into cells comprising the GS-X pump gene. The nucleic acid is antisense to either a portion or all of a plant GS-X pump gene, which gene is preferably *Arabidopsis AtMRP1*, *Arabidopsis AtMRP2* or a homolog thereof. The "isolated preparation of a nucleic acid" and the "portion" of the gene to which the nucleic acid is antisense, should be of a sufficient length so as to inhibit expression of the desired target gene. The actual length of the isolated preparation of the nucleic acid may vary, and will depend on the particular target gene and the region of that gene which is targeted. Typically, the isolated preparation of the nucleic acid will be at least about 15 contiguous nucleotides; more typically, it will be between about 15 and about 50 contiguous nucleotides, or it may even be more than 50 contiguous nucleotides in length.

As used herein, a sequence of a nucleic acid is "antisense" to a portion or all of a GS-X pump gene when the sequence of nucleic acid does not encode a GS-X polypeptide. Rather, the sequence which is being expressed in the cells is identical to the non-coding strand of the GS-X pump gene and thus, does not encode a GS-X pump polypeptide.

"Complementary," as used herein, refers to the subunit sequence complementarity between two nucleic acids, e.g., two DNA molecules. When a nucleotide position in both of the molecules is occupied by nucleotides normally capable of base pairing with each other, then the nucleic acids are considered to be complementary to each other at this position. Thus, two nucleic acids are complementary to each other when a substantial number (at least 50%) of corresponding positions in each of the molecules are occupied by nucleotides which normally base pair with each other (e.g., A:T and G:C nucleotide pairs).

In yet another aspect of the invention, there is provided an antibody directed against a plant GS-X pump, preferably AtMRP1 or AtMRP2, which antibody

is specific for the whole molecule or either the N-terminal or the C-terminal or internal portions of AtMRP1 or AtMRP2. Methods of generating such antibodies are well known in the art and are described, for example, in Harlow *et al.* (*supra*).

5 The present invention also provides for analogs of proteins or peptides encoded by *AtMRP1* or *AtMRP2*. Analogs can differ from naturally occurring proteins or peptides by conservative amino acid sequence differences or by modifications which do not affect sequence, or by both.

10 For example, conservative amino acid changes may be made, which although they alter the primary sequence of the protein or peptide, do not normally alter its function. Conservative amino acid substitutions typically include substitutions within the following groups:

15 glycine, alanine;  
valine, isoleucine, leucine;  
aspartic acid, glutamic acid;  
asparagine, glutamine;  
serine, threonine;  
lysine, arginine;  
phenylalanine, tyrosine.

20 Modifications (which do not normally alter primary sequence) include *in vivo*, or *in vitro* chemical derivatization of polypeptides, *e.g.*, acetylation, or carboxylation. Also included are modifications of glycosylation, *e.g.*, those made by modifying the glycosylation patterns of a polypeptide during its synthesis and processing or in further processing steps; *e.g.*, by exposing the polypeptide to enzymes which affect glycosylation, *e.g.*, mammalian glycosylating or deglycosylating enzymes. Also  
25 embraced are sequences which have phosphorylated amino acid residues, *e.g.*, phosphotyrosine, phosphoserine, or phosphothreonine.

Also included are polypeptides which have been modified using ordinary molecular biological techniques so as to improve their resistance to proteolytic degradation or to optimize solubility properties or to render them more suitable as a



therapeutic agent. Analogs of such polypeptides include those containing residues other than naturally occurring L-amino acids, *e.g.*, D-amino acids or non-naturally occurring synthetic amino acids. The peptides of the invention are not limited to products of any of the specific exemplary processes listed herein.

5           The invention further includes a transgenic plant comprising an isolated DNA encoding a plant GS-X pump polypeptide or a fragment thereof, capable of transporting a glutathionated compound across a biological membrane. The transgenic plant of the invention may comprise a transgene encoding a plant GS-X pump polypeptide or a fragment thereof, or it may comprise a transgene encoding a yeast GS-X pump polypeptide or a fragment thereof, which yeast transgene is expressed in the  
10   plant to yield a biologically active GS-X pump protein product. By way of example, there is provided herein in the experimental examples section a transgenic *Arabidopsis* plant comprising a yeast *YCF1* transgene, which when the transgene is expressed in the transgenic plant, confers upon the plant the ability to grow on media containing  
15   concentrations of heavy metal (cadmium) or organic xenobiotic (CDNB) that otherwise prevent of nontransgenic plants.

          The invention also includes a transgenic plant comprising an isolated DNA comprising the sequence of a plant GS-X pump polypeptide or a fragment thereof, which plant GS-X pump is capable of transporting a glutathionated compound  
20   across a membrane derived from a cell, wherein the sequence of the isolated DNA is positioned in an antisense orientation with respect to the direction of transcription of the DNA.

          Thus, included in the invention is a transgenic plant comprising an isolated DNA encoding a yeast *YCF1* or a fragment thereof, capable of transporting a  
25   glutathionated compound across a membrane derived from a cell.

          In addition, the invention includes a transgenic plant comprising an isolated DNA comprising the sequence of a yeast *YCF1* gene or a fragment thereof, wherein the sequence of the isolated DNA is positioned in an antisense orientation with respect to the direction of transcription of the DNA.

By "transgenic plant" as used herein, is meant a plant, the cells, the seeds and the progeny of which comprise a gene inserted therein, which gene has been manipulated to be inserted into the cells of the plant by recombinant DNA technology. The manipulated gene is designated as a "transgene."

5 By the term "nontransgenic but otherwise substantially homozygous wild type plant" as used herein, is meant a nontransgenic plant from which the transgenic plant was generated.

"Positioned in an antisense orientation with respect to the direction of transcription of the DNA" as used herein, means that the transcription product of the DNA, the resulting mRNA, does not encode a GS-X pump. Rather, the mRNA  
10 comprises a sequence which is complementary to an mRNA which encodes a GS-X pump.

If vacuolar transport rate limits xenobiotic detoxification and if the amount of GS-X pump is rate limiting on the overall rate of vacuolar uptake, transgenic  
15 plants with increased *YCF1*, *AtMRP1* or *AtMRP2* expression are expected to be more resistant to the toxic effects of glutathione-conjugable xenobiotics and capable of accumulating higher vacuolar conjugate levels than non-transgenic plants. The former property permits the sustained growth of transgenic plants in the presence of xenobiotic concentrations that would retard the growth of plants exhibiting normal levels of  
20 transporter expression. The latter property confers on the plants the ability for hyperaccumulation of glutathionated xenobiotics.

Increased resistance to xenobiotics has application in herbicide technology and plant growth in habitats polluted with organics. Hyperaccumulation has application in the extraction of organic pollutants from contaminated ground soils.

25 The closest known similar technologies to those described herein (a) involve the isolation of mutants or the engineering of plants in which the target for xenobiotic action is no longer sensitive, (b) involve the generation of mutants with elevated cellular levels of glutathione (GSH) or with increased glutathione-S-transferase activities, or (c) involve the application of chemical agents ("safeners") that

elevate GSH and/or glutathione-S-transferase levels or activities. These known technologies differ from the strategy proposed herein in three respects: (i) The utility of mutated target gene products is limited in its application to those xenobiotics that directly interact with the target in question. In contrast, the vacuolar GS-X pump is of broad substrate specificity. (ii) Technologies based on elevated cellular GSH levels or increased glutathione-S-transferase catalytic efficiencies are limited by the capacity of cells to subsequently metabolize and/or sequester the conjugates generated. The success of these latter technologies eventually depends on delivery of GSH-conjugates into the vacuole and in turn, depends on the activity of the vacuolar GS-X pump. (iii) Since the plant vacuole frequently constitutes 40-90% of total intracellular volume and the GS-X pump mediates the uptake of xenobiotics into this compartment, the potential for hyperaccumulation on a tissue weight basis is great. Hyperaccumulators may therefore be used for the fixation/sequestration of toxins and their removal from soils. None of the other known technologies have this characteristic.

The generation of transgenic plants comprising sense or antisense DNA having the sequence of a GS-X pump or a fragment thereof, may be accomplished by transformation of the plant with a plasmid encoding the desired DNA sequence. Suitable vectors include, but are not limited to, disarmed *Agrobacterium* tumor-inducing (Ti) plasmids containing a sense or antisense strand placed under the control of the strong constitutive CaMV 35S promoter or under the control of an inducible promoter (Lagrimini *et al.*, 1990, *supra*; van der Krol *et al.*, 1988, *Gene* 72:45-50). Methods for the generation of such constructs, plant transformation and plant regeneration are well known in the art once the sequence of the desired gene is known and are described, for example, in Ausubel *et al.* (1993, *Current Protocols in Molecular Biology*, Greene and Wiley, New York).

Suitable vector and plant combinations will be readily apparent to those of skill in the art and can be found, for example, in Maliga *et al.* (1994, *Methods in Plant Molecular Biology: A Laboratory Manual*, Cold Spring Harbor, New York).

Transformation of plants may be accomplished using the Agrobacterium-mediated leaf disc transformation method described by Horsch *et al.* (1988, *Leaf Disc Transformation, Plant Molecular Biology Manual A5:1*).

5 A number of procedures may be used to assess whether the transgenic plant comprises the desired DNA. For example, genomic DNA obtained from the cells of the transgenic plant may be analyzed by Southern blot hybridization or by PCR to determine the length and orientation of any inserted, transgenic DNA present therein. Northern blot hybridization analysis or PCR may be used to characterize mRNA transcribed in cells of the transgenic plant. In situations where it is expected that the  
10 cells of the transgenic plant express GS-X polypeptide or a fragment thereof, Western blot analysis may be used to identify and characterize polypeptides so expressed using antibody raised against the GS-X pump or fragments thereof. The procedures for performing such analyses are well known in the art and are described, for example, in Sambrook *et al.* (*supra*).

15 The transgenic plants of the invention are useful for the manipulation of xenobiotic detoxification, heavy metal detoxification, control of plant pathogens, control of plant coloration, herbicide metabolism and phytohormone metabolism. For example, a transgenic plant encoding an *AtMRP1* or an *AtMRP2* gene or an *AtMRP1*- or *AtMRP2*-related gene, or a yeast *YCF1* or *YCF1*-related gene, is useful for  
20 xenobiotic detoxification and heavy metal detoxification when grown on soil containing xenobiotics or heavy metals. Such plants are capable of removing xenobiotic toxins or heavy metals from the soil thereby generating soil which has reduced levels of compounds that are detrimental to the overall health of the environment.

25 Accordingly, the invention includes a method of removing xenobiotic toxins from soil comprising generating a transgenic plant having a transgene encoding a GS-X pump and planting the plant or the seeds of the plant in the soil wherein xenobiotic toxins in the soil are sequestered within the plant during growth of the plant in the soil.

Similarly, the invention includes a method of removing heavy metals from soil comprising generating a transgenic plant having a transgene encoding a GS-X pump and planting the plant or the seeds of the plant in the soil wherein heavy metals in the soil are sequestered within the plant during growth of the plant in the soil.

5 When the levels of xenobiotic toxins or heavy metals in the soil have been sufficiently reduced, the transgenic plant may be removed from the soil and destroyed or discarded in an environmentally safe manner. For example, the harvested plants can be reduced in volume and/or weight by thermal, microbial, physical or chemical means to decrease handling, processing and potential subsequent land filling costs (Cunningham *et al.*, 1996, *Plant Physiol.* 110:715-719). In the case of valuable  
10 metals, subsequent smelting and recovery of the metal may be cost-effective (Raskin, 1996, *Proc. Natl. Acad. Sci. USA* 93:3164-3166).

This technique of remediating soil is more efficient, less expensive and easier than most chemical or physical methods. The estimated costs of remediation are  
15 as follows: U.S. \$10-100 per cubic meter of soil for removal of volatile or water soluble pollutants by *in situ* remediation using plants; U.S. \$60-300 per cubic meter of soil for landfill or low temperature thermal treatment remediation of soil contaminated with the same compounds; and, U.S. \$200-700 per cubic meter of soil for remediation of soil contaminated with materials requiring special landfilling arrangements or high  
20 temperature thermal treatment (Cunningham *et al.*, 1995, *Trends Biotechnol.* 13:393-397).

Preferably, the transgene in the transgenic plant of the invention is *AtMRP1*, *AtMRP2*, *YCF1* or genes encoding fragments or analogs of *AtMRP1*, *AtMRP2* or *YCF1*, or the transgene is a gene which is related to *AtMRP1*, *AtMRP2*,  
25 *YCF1*.

The types of plants which are suitable for use in this method of the invention include, but are not limited to, high yield crop species for which cultivation practices have already been perfected, or engineered endemic species that thrive in the area to be remediated.

In certain situations, it may be necessary to prevent the removal of substances such as xenobiotic toxins and heavy metals from the soil. In such situations, transgenic plants are generated comprising a transgene comprising a GS-X pump sequence which is in the antisense orientation with respect to transcription. Such transgenes therefore serve to inhibit the function of a GS-X pump expressed in the plants thereby preventing removal of xenobiotics or heavy metals from the soil.

The production of plants having GS-X pump antisense sequences has application in the manipulation of plant/food coloration and in the diminution of organic xenobiotic (*e.g.*, herbicide) or heavy metal accumulation by crop species. For example, ingestion by animals or humans of low organic toxin/low heavy metal crops will likely contribute to an improvement in the overall health of animals and humans.

Accordingly, the invention includes a method of preventing the removal of xenobiotic toxins or heavy metals from soil comprising generating a transgenic plant having a transgene comprising a GS-X pump sequence which is in the antisense orientation with respect to transcription and planting the plant or the seeds of the plant in the soil, wherein removal of xenobiotics and heavy metals from the soil is prevented during growth of the plant in the soil.

The antisense sequences which are useful for the generation of transgenic plants having antisense GS-X pump sequences are those which will inhibit expression of a resident GS-X gene in the plant.

The types of plants which are suitable for use in this method of the invention using antisense sequences include, but are not limited to, plants for which anthocyanins contribute to flower, fruit or leaf coloration and food crops for which decreased organic xenobiotic and/or heavy metal accumulation is desirable.

In a similar manner to that described herein, a transgenic plant may be generated which exhibits increased accumulation and/or resistance to isoflavonoid alexins by introducing into the cells of the plant a transgene encoding a GS-X pump capable of transporting glutathionated isoflavonoid alexins into vacuoles in the plant, thereby isolating the isoflavonoid alexins from the cytoplasm of the cells of the plant.

Preferably, the transgene is *AtMRP1*, *AtMRP2*, *YCF1* or genes encoding fragments or analogs of *AtMRP1*, *AtMRP2* or *YCF1*, or the transgene is a gene which is related to *AtMRP1*, *AtMRP2* or *YCF1*.

5 The invention thus includes a method of generating a pathogen-resistant transgenic plant comprising introducing into the plant a transgene encoding a GS-X pump capable of transporting glutathionated isoflavonoid alexins into vacuoles in the plant.

The types of plants suitable for the introduction of the desired transgene include, but are not limited to, plants which are leguminous plants, for example, alfalfa,  
10 cashew nut, castor bean, faba bean, french bean, mung bean, pea, peanut, soybean and walnut.

As discussed herein, it has also been discovered in the present invention that the *Bz2* gene which encodes a glutathione-S-transferase, glutathionates anthocyanins and possibly other compounds for transport by the GS-X pump. The  
15 anthocyanin-derivatives so generated are subsequently transported across biological membranes by the vacuolar GS-X pump. Vacuolar anthocyanins are responsible for the red and purple hues of many plant organs (petals, leaves, stems, seeds, fruits, etc.). Vacuolar anthocyanins are found in most flowering plants. However, they are not solely responsible for plant coloration. Rather, plant coloration is determined by the  
20 relative amounts and combinations in which these various pigments are accumulated. Thus, it is possible to manipulate plant coloration by generating transgenic plants with increased (sense DNA) or decreased (antisense DNA) expression of the GS-X pump. Transgenic plants having GS-X pump sense sequences are expected to contain more red/purple pigmentation than their nontransgenic but otherwise homozygous  
25 counterparts and transgenic plants having GS-X pump antisense sequences are expected to contain less red/purple pigmentation and possibly more brown pigmentation than their nontransgenic but otherwise homozygous counterparts. The generation of such types of transgenic plants may be accomplished following the procedures described herein.

With respect to the aforementioned information regarding anthocyanins, it is important to note that accumulating evidence from studies of the MRP-subclass members from non-plant sources reveals that the group of transporters formerly referred to as GS-X pumps because of their affinity toward GS-conjugates, GSSG and cysteinyl leukotrienes, do not transport GS-conjugates exclusively (Ishikawa et al., 1997, Bioscience Reports 17:189-208). Investigation of the human MRP1 protein, cMOAT and ScYCF1 establish that these proteins are capable of transporting a broad range of compounds in addition to GS-conjugates and GSSG Jedlitschky et al., 1996, Cancer Res. 56:988-994; Paulusma et al., 1996, Science 271:1126-1128; Jansen et al., 1987, Hepatol. 7:71-76; Sathirakul et al., 1993, J. Pharmacol. Exp. Therap. 268:65-73). Thus, these proteins transport non-glutathionated compounds.

It has been discovered in the present invention that the plant proteins, AtMRP1 and AtMRP2, differ in their substrate preferences. For example, not only does AtMRP2 exhibit a much higher transport capacity than does AtMRP1, but AtMRP2 has the capacity to transport chlorophyll breakdown products in leaf senescence, which breakdown products are not glutathionated. Thus, according to the present invention, it is possible to manipulate plant coloration by changing the relative levels of expression of various members of this class of transporters in a plant cell. It is possible, using the information provided herein, to affect the rate of breakdown of chlorophyll, for example, by manipulating the expression of AtMRP2 in a plant cell.

In addition to the above, there is provided as part of the invention, *AtMRP1* and *AtMRP2* promoter sequences. By operably coupling the *AtMRP1* or *AtMRP2* promoters to other genes, it may be possible to confer on these other genes expression characteristics similar to those of *AtMRP1* or *AtMRP2*, namely, modulation by xenobiotics, plant pathogens, etc. The data which are presented herein include the promoter sequences of these genes, which promoter sequences are useful in a variety of applications in plants. For example, GS-X pump activity which is associated with herbicide metabolism (exemplified by organic xenobiotic transport), heavy metal sequestration (exemplified by cadmium transport), plant-pathogen interactions



(exemplified by vacuolar uptake of medicarpin), plant cell pigmentation (exemplified by transport of glutathionated anthocyanins) and plant hormone metabolism (exemplified by the transport of glutathionated auxins) may be examined as a result of the present invention. The present invention facilitates the identification of plants and  
5 cells therein which are capable of GS-X pump activity, and further facilitates the exploitation of plant cell GS-X pump activity for the purpose of affecting plant function with respect to herbicide metabolism, heavy metal sequestration, plant-pathogen interactions, plant cell pigmentation and plant hormone metabolism.

The invention includes an isolated DNA comprising a plant GS-X pump  
10 promoter sequence capable of driving expression of a plant GS-X pump gene, which gene is capable of transporting a glutathionated compound across a biological membrane. Preferably, the membrane is derived from a cell.

Preferably, the isolated DNA comprising a plant GS-X pump promoter  
15 sequence is at least about 40% homologous to at least one of the *AtMRP1* or *AtMRP2* promoter sequences presented herein in Figures 23 and 24, respectively. More preferably, the isolated DNA comprising a plant GS-X pump promoter sequence is at least about 50%, even more preferably, at least about 60%, yet more preferably, at least about 70%, even more preferably, at least about 80%, yet more preferably, at least  
20 about 90% homologous, and more preferably, at least about 99% homologous to at least one of *AtMRP1* or *AtMRP2* promoter sequences presented herein in Figures 23 and 24, respectively. Most preferably, the isolated DNA comprising a plant GS-X pump promoter sequence is *Arabidopsis AtMRP1* or *AtMRP2* as shown in Figures 1 and 2, respectively.

Thus, the invention should be construed to include isolated DNA  
25 sequences comprising promoter sequences which in their natural form drive expression of genes which encode *Arabidopsis AtMRP1* and *AtMRP2* and *Arabidopsis AtMRP1* and *AtMRP2*-related genes. Once armed with the present invention, it is a simple matter to isolate sequences which are related to those shown in Figures 1 and 2. For example, conventional hybridization technology and/or PCR technology may be

employed, primers may be designed using the sequences provided herein, data bases may be searched and the like. Procedures for the isolation of promoter sequences which are related to those described herein are described in Sambrook et al. (1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY) and in Ausubel  
5 et al. (1993, *Current Protocols in Molecular Biology*, Greene and Wiley, New York).

By the term "promoter sequence" as used herein, is meant a DNA sequence which is required for expression of a gene which is operably linked thereto. In some instances, this sequence may be a core promoter sequence and in other instances, this sequence may also include an enhancer sequence and other regulatory  
10 elements which are required for expression of the gene in a tissue-specific manner. Thus, a promoter sequence must include an RNA polymerase binding site and may include appropriate transcription factor binding sites as are necessary for activation of transcription and expression of the gene to which the promoter sequence is attached at the 5' end of the gene.

15 Typically, the promoter sequence of the invention comprises at least about 150 bp in length. More typically, the promoter sequence comprises at least about 300 bp in length. More typically, the promoter sequence comprises at least about 400 bp, even more typically, at least about 500 bp, yet more typically, at least about 600 bp, even more typically, at least about 800 bp, yet more typically, at least about 1000 bp  
20 and even more typically, at least about 1200 or more bp in length.

The promoter sequence of the invention may also comprise discrete sequences (elements) which function to regulate the activity of the promoter. Frequently, such elements respond to the presence or absence of environmental factors, thereby controlling gene expression in direct response to factors which are associated  
25 with the environmental milieu of the plant. The response of the plant to these factors affects the overall well-being of the plant. Elements which may be present in the promoter sequence of the invention include, but are not limited to, a Myb recognition sequence, a xenobiotic regulatory element, an antioxidant response element, a bZIP recognition sequence, and the like.

Plants from which *AtMRP1*- or *AtMRP2*-related genes and therefore promoter sequences, may be isolated include any plant in which the GS-X pump is found, including, but not limited to, soybean, castor bean, maize, petunia, potato, tomato, sugar beet, tobacco, oats, wheat, barley, pea, faba bean and alfalfa.

5 The invention further includes a vector comprising a plant GS-X pump promoter sequence operably fused to a reporter gene and capable of driving expression of the reporter gene. The procedures for the generation of a vector comprising a plant GS-X pump promoter sequence are well known in the art once the sequence of the gene is known, and are described, for example, in Sambrook et al. (*supra*). Suitable vectors  
10 include, but are not limited to, disarmed *Agrobacterium* tumor-inducing (Ti) plasmids (e.g., pBIN19) (Lagrimini et al., 1990, *Plant Cell* 2:7-18; Bevan, 1984, *Nucl. Acids Res.* 12:8711-8721).

Also included in the invention is a cell comprising a plant GS-X pump promoter sequence operably fused to a reporter gene. The procedures for the generation  
15 of a cell encoding a plant GS-X pump or fragment thereof, are well known in the art once the sequence of the gene is known, and are described, for example, in Sambrook et al. (*supra*). Suitable cells include, but are not limited to, plant cells, yeast cells, bacterial cells, mammalian cells, and baculovirus-infected insect cells. In addition, plant cells transformed with the promoter/reporter gene construct, for the purpose of  
20 assessing the effect of various compounds on promoter activity are also contemplated in the invention. Normal plant cells and those plant cells having increased resistance to and increased capacity for heavy metal accumulation, increased resistance to organic xenobiotics and increased capacity for organic xenobiotic accumulation or altered coloration, which cells comprise the promoter sequence of the invention operably fused  
25 to a reporter gene, are all contemplated as part of the invention. When the promoter is fused to a reporter gene, the promoter is said to be operably linked to the reporter gene.

A "reporter gene" as used herein, is one which when expressed in a cell, results in the production of a detectable product in the cell. The level of expression the

product in the cell is proportional to the activity of the promoter sequence which drives expression of the reporter gene.

By describing two nucleic acid sequences as "operably linked" as used herein, is meant that a single-stranded or double-stranded nucleic acid moiety  
5 comprises each of the two nucleic acid sequences and that the two sequences are arranged within the nucleic acid moiety in such a manner that at least one of the two nucleic acid sequences is able to exert a physiological effect by which it is characterized upon the other.

Suitable reporter genes include, but are not limited to,  $\beta$ -glucuronidase  
10 (GUS) and green fluorescent protein (GFP), although any reporter gene capable of expression and detection in plant cells which are either known or heretofore unknown, may be fused to the plant GS-X promoter sequences of the invention.

The invention further includes a transgenic plant comprising an isolated DNA comprising a plant GS-X pump promoter sequence as defined herein.

15 The generation of transgenic plants comprising a plant GS-X pump promoter sequence operably fused to a reporter gene, may be accomplished by transformation of the plant with a plasmid comprising the desired DNA sequence. Suitable vectors include, but are not limited to, disarmed *Agrobacterium* tumor-inducing (Ti) plasmids (Lagrimini *et al.*, 1990, *supra*; van der Krol *et al.*, 1988, *Gene*  
20 72:45-50). Methods for the generation of such constructs, plant transformation and plant regeneration are well known in the art once the sequence of the desired nucleic acid is known and are described, for example, in Ausubel *et al.* (1993, *Current Protocols in Molecular Biology*, Greene and Wiley, New York).

Suitable vector and plant combinations will be readily apparent to those  
25 of skill in the art and can be found, for example, in Maliga *et al.* (1994, *Methods in Plant Molecular Biology: A Laboratory Manual*, Cold Spring Harbor, New York).

Transformation of plants may be accomplished using the *Agrobacterium*-mediated leaf disc transformation method described by Horsch *et al.* (1988, *Leaf Disc Transformation, Plant Molecular Biology Manual* A5:1).

A number of procedures may be used to assess whether the transgenic plant comprises the desired DNA. For example, genomic DNA obtained from the cells of the transgenic plant may be analyzed by Southern blot hybridization or by PCR to determine the length and orientation of any inserted, transgenic DNA present therein.

5 Northern blot hybridization analysis or RT-PCR may be used to characterize mRNA transcribed in cells of the transgenic plant. In situations where it is expected that the cells of the transgenic plant express GS-X polypeptide or a fragment thereof, Western blot analysis may be used to identify and characterize polypeptides so expressed using antibody raised against the GS-X pump or fragments thereof. The procedures for

10 performing such analyses are well known in the art and are described, for example, in Sambrook et al. (*supra*).

The transgenic plants of the invention are useful for the examination of xenobiotic detoxification, heavy metal detoxification, control of plant pathogens, control of plant coloration, herbicide metabolism and phytohormone metabolism. For

15 example, a transgenic plant comprising an *AtMRP1* or an *AtMRP2* promoter sequence fused to a reporter gene is useful for the examination of xenobiotic detoxification and heavy metal detoxification when grown on soil having xenobiotic toxins or heavy metals. Such plants are useful to an understanding of the mechanisms by which GS-X pump gene expression is activated and are therefore useful for the eventual generation

20 of plants which are capable of removing xenobiotic toxins or heavy metals from the soil thereby generating soil which has reduced levels of compounds that are detrimental to the overall health of the environment.

The types of plants which are suitable for use include, but are not limited to, high yield crop species for which cultivation practices have already been

25 perfected, or engineered endemic species that thrive in the area to be remediated. In addition plants for which anthocyanins contribute to flower or leaf coloration and food crops for which decreased organic xenobiotic and/or heavy metal accumulation is desirable are also suitable for use in the invention. Further useful plants are those in which it is desirable that they are capable of increased accumulation and/or resistance

to isoflavonoid alexins. Plants for which pathogen resistance is desired are also useful in the invention. Such plants include, but are not limited to, plants which are leguminous plants, for example, alfalfa, cashew nut, castor bean, faba bean, french bean, mung bean, pea, peanut, soybean and walnut. In addition, plants for which it is desirable to manipulate plant coloration are also useful in the invention.

The promoter sequences of *Arabidopsis* GS-X pump genes *AtMRP1* and *AtMRP2* are shown in Figures 23 and 24, respectively. The following should be noted. bZIP transcription factor recognition elements have the sequences CACGTG or TGACG(T/C). One of these is present in the *AtMRP2* promoter sequence, but none are present in the *AtMRP1* promoter sequence. Myb transcription factor recognition elements having the sequences A(a/D)(a/D)C(G/C) and AGTTAGTTA, wherein a/D = A, G or T with A being preferred, are present in the *AtMRP1* promoter sequence, but are not present in the *AtMRP2* promoter sequence. Xenobiotic regulatory elements (XREs) having the core sequence GCGTG are found in multiple copies in the promoters of cytochrome P450 monooxygenase genes and glutathione S-transferase genes (Rushmore et al., 1993, J. Biol. Chem. 268:11475-11478). One XRE is found in the promoter sequence of *AtMRP1*. Antioxidant response elements (AREs) consist typically of two core sequences GTGACA(A/T)(A/T)GC (SEQ ID NO:11) that are binding sites for Activator Protein-1 (AP-1) transcription factor complex (Daniel, 1993, CRC Crit. Rev. Biochem 25:173-207; Friling et al., 1992, Proc. Natl. Acad. Sci. USA 89:668-672). There is only one ARE in the *AtMRP1* promoter sequence shown in Figure 23. It has been proposed that GST genes containing an ARE are induced by electrophiles and conditions that generate oxidative stress (Daniel, *supra*). RNA instability determinants having the sequence ATTTA have been found in several plant GSTs. These sequences, considered to target RNAs for degradation by RNases are usually found in the 3'-UTRs of genes (Takahashi et al., 1992, Proc. Natl. Acad. Sci. USA 89:56-59). Several of these sequences are found in both the *AtMRP1* and *AtMRP2* promoter sequences presented herein. However, it is not clear whether these sequences merely reflect the AT-richness of the sequences.

To assess GS-X pump gene expression in a plant cell whether the cell is contained within a plant or whether the cell is separated from the plant, a plasmid may be generated which comprises the  $\beta$ -glucuronidase (GUS) reporter gene fused to a plant GS-X promoter sequence. Preferably, the promoter sequence is either *AtMRP1* or *AtMRP2*. The appropriate restriction fragment is subcloned into the GUS expression vector pBI101.3 (Jefferson et al., 1987, EMBO J., 6:3901-3907). After confirming the correct reading frame by sequencing, *Agrobacterium* or any other suitable vector, is transformed with the expression construct and is then used to transform the plant, or the cells thereof (Valvekens et al., 1988, Proc. Natl. Acad. Sci. USA 85:5536-5540).

Expression of GUS may be localized histochemically by staining with 5-bromo-4-chloro-3-indoyl  $\beta$ -D-glucuronide (X-Gluc) (Jefferson et al., *supra*). Sections are obtained from the plant, they are incubated in X-Gluc, cleared by boiling in ethanol and are examined under the microscope. To eliminate or enumerate complications arising from the transfer of GUS reaction product between cells, the distribution of GUS expression is then further examined both immunologically and biochemically.  $\beta$ -glucuronidase protein is assessed using standard dot-blotting and immunolocalization techniques (Harlow et al., 1988, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, NY) using rabbit anti- $\beta$ -glucuronidase serum (Clontech). Direct estimates of GUS activity are made fluorimetrically using 4-methyl-umbelliferyl glucuronide as substrate (Jefferson et al., *supra*) after dissection and extraction of explants.

GUS reporter gene analyses enable examination of plant responses to oxidative stress and pathogens as well as herbicides. In addition, GUS reporter gene analyses enable tests of whether certain pigment-rich cell types also exhibit high levels of *AtMRP* expression.

The *AtMRP1* and *AtMRP2* promoter sequences are also useful for manipulating the expression of other genes in plants in that, transgenic plants may be generated which contain a desired plant gene operably fused to a GS-X pump promoter

sequence. The GS-X pump promoter sequence may be an *AtMRP1* or an *AtMRP2* promoter sequence or a *YCF1* promoter sequence positioned in an orientation such that the promoter sequence drives expression of the desired gene. The desired gene may be a plant or a non-plant gene. The generation of such transgenic plants confers upon the plants the ability to respond to the presence of xenobiotics and other compounds which influence the promoter activity

In considering transport substrates for GS-X pumps, the status of GSSG as an endogenous GS-conjugate (of GSH with itself) and its involvement in cellular responses to active oxygen species (AOS) should not be overlooked. The sulfhydryl group of GSH confers strong nucleophilicity and the facility for reacting with AOS, such as superoxide radicals ( $O_2^{\cdot -}$ ), hydroxyl radicals ( $OH^{\cdot}$ ) and hydrogen peroxide. GSH is found in the majority of eukaryotes but in prokaryotes (eubacteria) it appears to be restricted to the cyanobacteria and purple bacteria (Fahey and Sundquist 1991, Adv. Enzymol. Relat. Mol. Biol. 64:1-53). Since the cyanobacteria are considered to be the first group of organisms capable of oxygenic photosynthesis and these and the purple bacteria probably gave rise to plant chloroplasts and mitochondria, respectively, it has been proposed that the emergence of the capacity for GSH biosynthesis was associated with the appearance of oxygenic and oxytrophic metabolism (approximately  $4 \times 10^9$  years ago) to combat the attendant problem of AOS production. Most, if not all, of the factors known to elicit GST induction - pathogen attack, heavy metals, certain organic xenobiotics, wounding and ethylene - promote AOS production (Inze and Montagu 1995, Current Opinion in Biotech. 6:153-158). Intriguing, therefore, is the possibility that GS-X pumps arose from the need to detoxify AOS and the products of their action.

The feasibility of such a scheme has yet to be investigated systematically but a number of disparate observations are at least consistent with a close connection between oxidative stress and GS-X pump function: (i) All identified MRP-subclass transporters, including *AtMRP1* and *AtMRP2* recognize GSSG as a substrate. Studies of GS-X pumps originated from the discovery of ATP-dependent



GSSG efflux from erythrocytes (Srivastava and Beutler 1969, J. Biol. Chem. 244:9-16). (ii) In *S. cerevisiae*, overexpression of yAP1, a bZIP transcription factor, not only activates the YCF1 and GSH1 genes (Wemmie et al 1994, *supra*; Wu and Moye-Röwley 1994, *supra*), the latter of which encodes  $\gamma$ -glutamylcysteine synthetase, but also a panoply of oxidoreductases (DeRisi et al 1997, Science 278:680-686). Of the 17 genes whose mRNA levels are found to be increased by more than threefold on DNA microarrays by yAP1, more than two-thirds contain canonical upstream yAP1-binding sites (TTACTAA or TGACTAA), five bear homology to aryl-alcohol oxidoreductases and four to the general class of dehydrogenases/oxidoreductases (DeRisi et al 1997, *supra*). In view of the capacity of yAP1 overexpression to confer increased resistance to hydrogen peroxide, o-phenanthroline and heavy metals (Hirata et al 1994, Mol. Gen. Genet. 242:250-257), the fact that an appreciable fraction of the yAP1-regulated target genes identified against the yeast genome project database are oxidoreductases and coregulated with both YCF1 and GSH1, suggests that all of these genes play a protective role during oxidative stress. (iii) Two particularly harmful and early effects of AOS production are membrane lipid peroxidation, and oxidative DNA damage which yield highly toxic 4-hydroxyalkenals (Esterbauer et al 1991, Biochem. J. 208:129-140) and base propanols (Berhane et al 1994, Proc. Natl. Acad. Sci. USA 91:1480-1484), respectively. Although such  $\alpha,\beta$ -unsaturated aldehydes (and their GS-conjugates) have not yet been screened against the GS-X pumps from plant sources, they are established substrates for mammalian GSTs (Berhane et al 1994, *supra*) and their glutathionated derivatives are transported at high efficiency by mammalian GS-X pumps (Ishikawa 1989, J. Biol. Chem. 264:17343-17348).

There is therefore also included in the invention a method of alleviating oxidative stress in a plant comprising introducing into the cells of the plant DNA encoding a GS-X pump.

The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of

illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

5                   Experimental Examples

The experimental examples described herein provide procedures and results for the isolation and characterization of yeast *YCF1* and *Arabidopsis AtMRP1* and *AtMRP2* genes, gene products and various functions ascribed thereto. Further there is described data which establish that the *Bz2* gene product exerts its effects on  
10                   plant coloration via the GS-X pump.

The data which are now described establish that *YCF1* is a vacuolar glutathione S-conjugate pump. The data establish that *YCF1* is a membrane protein which is responsible for catalyzing MgATP-dependent, uncoupler-insensitive uptake of glutathione S-conjugates into the vacuole of wild type *S. cerevisiae*.

15                   *YCF1* encodes a protein responsible for resistance of yeast to the effects of cadmium. However, the mechanism by which resistance to  $Cd^{2+}$  is effected was not understood until the present invention. The data presented herein demonstrate that *YCF1* confers  $Cd^{2+}$  resistance to yeast by effecting transport of  $Cd^{2+}$  out of the cytosol via a *YCF1* encoded vacuolar glutathione S-conjugate pump. Further, since *YCF1*  
20                   confers resistance to  $Cd^{2+}$  through the transport of Cd.GS complexes or derivatives thereof, it is likely also capable of transporting other metal.GS-complexes. Examples of these other complexes include, but are not limited to, mercury (Hg), zinc (Zn), platinum (Pt) and arsenic (Ar). Both  $Hg^{2+}$  and  $Zn^{2+}$  form complexes with GSH which are analogous to those formed by  $Cd^{2+}$  (Li *et al.*, 1954, *J. Am. Chem. Soc.* 76:225-229; Kapoor *et al.*, 1965, *Biochem. Biophys. Acta* 100:376-383; Perrin *et al.*, 1971,  
25                   *Biochem. Biophys. Acta* 230:96-104). In addition, MRP1 eliminates the  $Pt^{2+}$  glutathione complex bis(glutathionato)platinum from cancer cells (Ishikawa *et al.*, 1994, *J. Biol. Chem.* 269:29085-29093). Further, the MRP1 gene is overexpressed in cisplatin-resistant human leukemia HL-60 cells, which overexpression is associated

with increased resistance to arsenite (Ishikawa *et al.*, 1996, *J. Biol. Chem.* 271:14981-14988). Both  $Hg^{2+}$  and  $As^{3+}$  are common environmental contaminants and  $Zn^{2+}$  is an essential micronutrient.

According to the results of the present study, vacuolar membrane vesicles from wild type *S. cerevisiae* catalyze high rates of MgATP-dependent, uncoupler-insensitive S-conjugate transport, and the kinetics of the transporter involved are similar to those of the mammalian and plant vacuolar GS-X pumps. In addition, vacuole-deficient mutants of *S. cerevisiae* exhibit markedly increased sensitivity to cadmium, leading to the belief that one requirement for efficient elimination or detoxification of this metal is maintenance of a sizable vacuolar compartment.

It is known that *S. cerevisiae* yAP-1 transcription factor transcriptionally activates both the *YCF1* gene and the *GSH1* gene (Wemmie *et al.*, 1994, *J. Biol. Chem.* 269:32592-32597; Wu *et al.*, 1994, *Mol. Cell. Biol.* 14:5832-5839). Since *GSH1* encodes  $\gamma$ -glutamylcysteine synthetase, an enzyme critical for GSH synthesis, expression of the *YCF1* gene and fabrication of one of the precursors for transport by the GS-X pump are coordinately regulated.

In the first set of experiments described below, transport of the model compounds DNP-GS and bimane-GS by isolated membrane vesicles and intact cells was examined.

#### Yeast Strains and Plasmids

Two strains of *S. cerevisiae* were used in these studies: DTY165 (*MAT $\alpha$  ura3-52 his6 leu2-3,-112 his3- $\Delta$ 200 trp1-901 lys2-801 suc2- $\Delta$* ) and the isogenic *ycf1 $\Delta$*  mutant strain, DTY167 (*MAT $\alpha$  ura 3-52 his6 leu2-3,-112 his3- $\Delta$ 200 trp 1-901 lys2-801 suc2- $\Delta$ , *ycf1::hisG*). The strains were routinely grown in rich (YPD) medium, or, when transformed with plasmid containing functional *YCF1* gene, in synthetic complete medium (Sherman *et al.*, 1983, *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory, New York) or AHC medium (Kim *et al.*, 1994, *supra*) lacking the appropriate amino acids. *Escherichia coli* strains XL1-blue (Stratagene)*

and DH11S were employed for the construction and maintenance of plasmid stocks (Ausubel *et al.*, 1987, *Current Protocols in Molecular Biology*, Wiley, New York).

Plasmid pYCF1-HA, encoding epitope-tagged *YCF1*, was constructed in several steps. A 1.4-kb *SalI-HindIII* fragment, encompassing the carboxyl-terminal segment of the open reading frame of *YCF1*, from pIBIYCF1 (Szczypka *et al.*, 1994, *supra*), was subcloned into pBluescript KS<sup>-</sup>. Single-stranded DNA was prepared and used as template to insert DNA sequence encoding the human influenza hemagglutinin 12CA5 epitope immediately before the termination codon of the *YCF1* gene by oligonucleotide-directed mutagenesis. The sequence of the primer for this reaction, with the coding sequence for the 12CA5 epitope underlined, was 5'-  
GTTTCACAGTTTAAAGCGTAGTCTGGGACGTCGTATGGGTAATTTTCATTG  
ACC-3' (SEQ ID NO:12). After confirming the boundaries and fidelity of the HA-tag coding region by DNA sequencing, the 1.4-kb *SalI-HindIII* DNA fragment was exchanged with the corresponding wild type segment of pJAW50 (Wemmie *et al.*, 1994, *supra*) to generate pYCF1-HA.

#### Isolation of Vacuolar Membrane Vesicles

For the routine preparation of vacuolar membrane vesicles, 15 ml of stationary phase cultures of DTY165 or DTY167 were diluted into 1-liter volumes of fresh YPD medium, grown for 24 hours at 30°C to an OD<sub>600 nm</sub> of approximately 0.8 and collected by centrifugation. After washing with distilled water, the cells were converted to spheroplasts with Zymolyase 20T (ICN) (Kim *et al.*, 1994, *supra*) and intact vacuoles were isolated by flotation centrifugation of spheroplast lysates on Ficoll 400 step gradients as described by Roberts *et al.* (1991, *Methods. Enzymol.* 194:644-661). Both the spheroplast lysis buffer and Ficoll gradients contained 2 mg/ml bovine serum albumin, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 1 mM PMSF to minimize proteolysis. The resulting vacuole fraction was vesiculated in 5 mM MgCl<sub>2</sub>, 25 mM KCl, 10 mM Tris-Mes (pH 6.9) containing 2 mg/ml bovine serum albumin, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 1 mM PMSF, pelleted by centrifugation at 37,000 x g for 25 min, and resuspended

in suspension medium (1.1 M glycerol, 2 mM dithiothreitol, 1 mM Tris-EGTA, 2 mg/ml bovine serum albumin, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM PMSF, 5 mM Tris-Mes, pH 7.6) (Kim *et al.*, 1995, *J. Biol. Chem.* 270:2630-2635).

5 In experiments involving cadmium transport, dithiothreitol and EGTA were removed from the suspension medium to prevent the attenuation of YCF1-dependent  $\text{Cd}^{2+}$  transport otherwise exerted by these compounds. Vesiculated vacuolar membranes were subjected to three cycles of 50-fold dilution into simplified suspension medium (1.1 M glycerol, 5 mM Tris-Mes, pH 8.0), centrifugation at  
10 100,000 x g for 35 minutes and resuspension in the same medium before use.

For the experiment shown in Figure 4, 1 ml of partially purified vacuolar membrane vesicles (1.1-1.2 mg of protein), prepared by Ficoll flotation, were subjected to further fractionation by centrifugation through a 30-ml linear 10-40% (w/v) sucrose density gradient at 100,000 x g for 2 hours. Successive fractions were  
15 collected from the top of the centrifuge tube and, after determining sucrose concentration refractometrically, the fractions were diluted with suspension medium. The diluted fractions were sedimented at 100,000 x g and resuspended in 100-µl aliquots of suspension medium for assay. For the immunoblots shown in Figure 5 and the marker enzyme analyses shown in Table 4, crude microsomes were prepared by  
20 homogenization of spheroplasts in suspension medium and the sedimentation of total membranes at 100,000 x g for 35 minutes.

Microsomes and purified vacuolar membranes that were to be employed for SDS-polyacrylamide gel electrophoresis and immunoblotting were washed free of bovine serum albumin by three rounds of suspension in suspension  
25 medium minus bovine serum albumin and centrifugation at 100,000 x g for 35 minutes. The final membrane preparations were either used immediately or frozen in liquid nitrogen and stored at -85°C.

#### Measurement of Marker Enzyme Activities

$\alpha$ -Mannosidase was determined according to Opheim (1978, *Biochem. Biophys. Acta* 524:121-125) using *p*-nitrophenyl- $\alpha$ -D-mannopyranoside as substrate. NADPH-cytochrome *c* reductase was estimated as FMN-promoted reduction of NADPH (Kubota *et al.*, 1977, *J. Biol. Chem.* 81:197-201). GDPase was measured as the rate of liberation of  $P_i$  from GDP (Yanagisawa *et al.*, 1990, *J. Biol. Chem.* 265:19351-19355) in reaction buffer containing 0.05% (w/v) Triton X-100. V-ATPase, F-ATPase, and P-ATPase were assayed as bafilomycin  $A_1$  (1  $\mu$ M), azide (1 mM), and vanadate (100  $\mu$ M) inhibited ATPase activity, respectively, at pH 8.0 (V-ATPase, F-ATPase) or pH 6.5 (P-ATPase) (Rea and Turner, 1990, *Methods Plant Biochem.* 3:385-405).

#### Measurement of DNP-GS Uptake

Unless otherwise indicated, [ $^3$ H]DNP-GS uptake was measured at 25°C in 200  $\mu$ l reaction volumes containing 3 mM ATP, 3 mM  $MgSO_4$ , 5  $\mu$ M gramicidin-D, 10 mM creatine phosphate, 16 units/ml creatine kinase, 50 mM KCl, 1 mg/ml bovine serum albumin, 400 mM sorbitol, 25 mM Tris-Mes (pH 8.0), and 66.2  $\mu$ M [ $^3$ H]DNP-GS (8.7 mCi/mmol) (Li *et al.*, 1995, *supra*). Gramicidin D was included in the uptake medium to abolish the  $H^+$  electrochemical potential difference ( $\Delta\mu_{H^+}$ ) that would otherwise be established by the V-ATPase in medium containing MgATP. Uptake was initiated by the addition of vacuolar membrane vesicles (10-15  $\mu$ g of membrane protein), brief mixing of the samples on a vortex mixer and uptake was then allowed to proceed for 1-60 minutes. Uptake was terminated by the addition of 1 ml of ice-cold wash medium (400 mM sorbitol, 3 mM Tris-Mes, pH 8.0) and vacuum filtration of the suspension through prewetted Millipore HA cellulose nitrate membrane filters (pore diameter, 0.45  $\mu$ m). The filters were rinsed twice with 1 ml of ice-cold wash medium and air-dried, and radioactivity was determined by liquid scintillation counting in BCS mixture (Amersham Corp.). Nonenergized [ $^3$ H]DNP-GS uptake and extravesicular solution trapped on the filters were enumerated by the same procedure except that ATP and  $Mg^{2+}$  were omitted from the uptake medium.

#### Fluorescence Microscopy

Cells were grown in YPD medium for 24 hours at 30°C to an OD<sub>600 nm</sub> of approximately 1.4, and 100 µl aliquots of the suspensions were transferred to 15 ml volumes of fresh YPD medium containing 100 µM *syn*-(ClCH<sub>2</sub>CH<sub>3</sub>)-1,5-diazabicyclo-[3.3.0]-octa-3,6-dione-2,8-dione (monochlorobimane) (Kosower *et al.*, 1980, *J. Am. Chem. Soc.* 102:4983-4993). After incubation for 6 hours, the cells were pelleted by centrifugation, washed twice with YPD medium lacking monochlorobimane, and viewed without fixation under an Olympus BH-2 fluorescence microscope equipped with a BP-490 UV excitation filter, AFC-0515 barrier filter, and Nomarski optics attachment.

#### Electrophoresis and Immunoblotting

Membrane samples were subjected to one-dimensional SDS-polyacrylamide gel electrophoresis on 7-12% (w/v) concave exponential gradient gels after delipidation with acetone:ethanol (Parry *et al.*, 1989, *J. Biol. Chem.* 264:20025-20032). The separated polypeptides were electrotransferred to 0.45 µm nitrocellulose filters at 60 V for 4 hours at 4°C in a Mini Trans-Blot transfer cell (Bio-Rad) and reversibly stained with Ponceau-S (Rea *et al.*, 1992, *Plant Physiol.* 100:723-732). The filters were blocked and incubated overnight with mouse anti-HA monoclonal antibody (20 µg/ml) (Boehringer-Mannheim). Immunoreactive bands were visualized by reaction with horseradish peroxidase-conjugated goat anti-mouse IgG (1/1000 dilution) (Boehringer-Mannheim) and incubation in buffer containing H<sub>2</sub>O<sub>2</sub> (0.03% w/v), diaminobenzidine (0.6 mg/ml) and NiCl<sub>2</sub> (0.03% w/v) (Rea *et al.*, 1992, *supra*).

#### Purification of Cadmium-Glutathione Complexes

Singly radiolabeled <sup>109</sup>Cd.GS<sub>n</sub> and doubly radio-labeled <sup>109</sup>Cd[<sup>3</sup>H].GS<sub>n</sub> complexes were prepared by sequential gel-filtration and anion-exchange chromatography of the reaction products generated by incubating 20 mM <sup>109</sup>CdSO<sub>4</sub> (78.4 mCi/mmol) with 40 mM GSH or 40 mM [<sup>3</sup>H]GSH (240 mCi/mmol) in 15 ml 10 mM phosphate buffer (pH 8.0) containing 150 mM KNO<sub>3</sub> at 45°C for 24 hours. For gel-filtration, 2 ml aliquots of the reaction mixture were applied to a column (40 x 1.5 cm ID) packed with water-equilibrated Sephadex G-15, eluted with

deionized water and  $^{109}\text{Cd}$  and/or  $^3\text{H}$  in the fractions was measured by liquid scintillation counting. The fractions encompassed by each of the two  $^{109}\text{Cd.GS}_n$  peaks identified were pooled, lyophilized and redissolved in 4 ml of loading buffer (5 mM Tris-Mes, pH 8.0). For anion-exchange chromatography, 0.5 ml aliquots of the resuspended lyophilizates from gel-filtration chromatography were applied to a Mono-Q HR5/5 column (Pharmacia) equilibrated with the same buffer. Elution was with a linear gradient of NaCl (0.5 ml/minute; 0-500 mM) dissolved in loading buffer. The individual fractions corresponding to the major peaks of  $^{109}\text{Cd}$  obtained from the Mono-Q column (one each for the peaks resolved by gel-filtration chromatography) were pooled, lyophilized and resuspended in 4 ml deionized water after liquid scintillation counting. Buffer salts were removed before transport measurements or mass spectrometry by passing the samples down a column (120 x 1.0 cm ID) packed with water-equilibrated Sephadex G-15.

#### Measurement of $^{109}\text{Cd}^{2+}$ Uptake

MgATP-energized, uncoupler-insensitive  $^{109}\text{Cd}^{2+}$  uptake by vacuolar membrane vesicles was measured at 25°C in 200 µl reaction volumes containing 3 mM ATP, 3 mM  $\text{MgSO}_4$ , 5 µM gramicidin-D, 10 mM creatine phosphate, 16 units/ml creatine kinase, 50 mM KCl, 400 mM sorbitol, 25 mM Tris-Mes (pH 8.0) and the indicated concentrations of  $^{109}\text{CdSO}_4$ , GSH or  $^{109}\text{Cd}$ - and/or  $^3\text{H}$ -labeled purified Cd.GS<sub>n</sub> complexes as described herein except that the wash media contained 100 µM  $\text{CdSO}_4$  in addition to sorbitol (400 mM) and Tris-Mes (3 mM, pH 8.0).

#### Pretreatment of DTY165 Cells with $\text{Cd}^{2+}$ or 1-Chloro-2,4-

#### dinitrobenzene

For studies on the inducibility of *YCF1* expression and *YCF1*-dependent transport, DTY165 cells were grown in YPD medium (Sherman *et al.*, 1983, *supra*) for 24 hours at 30°C to an  $\text{OD}_{600\text{ nm}}$  of 1.0-1.2, pelleted by centrifugation and resuspended in fresh YPD medium containing  $\text{CdSO}_4$  (200 µM) or 1-chloro-2,4-dinitrobenzene (CDNB). After washing in distilled water, total RNA was extracted and vacuolar membrane vesicles were prepared from the pretreated cells.



Control RNA and membrane samples were prepared from DTY165 cells treated in an identical manner except that  $\text{CdSO}_4$  and CDNB were omitted from the second incubation cycle.

#### RNase Protection Assays

5  $\text{Cd}^{2+}$  and CDNB-elicited increases in *YCF1* mRNA levels were assayed by RNase protection using 18S rRNA as an internal control. *YCF1*-specific probe was generated by PCR amplification of the full-length *YCF1::HA* gene, encoding human influenza hemagglutinin 12CA5 (HA) epitope-tagged *YCF1*, using plasmid pYCF1-HA as template. The forward *YCF1*-specific primer and backward primer containing  
10 the HA-tag coding sequence had the sequences 5'-AAACTGCAGATGGCTGGTAATCTTGTTTC-3' (SEQ ID NO:13) and 5'-GCCTCTAGATCAAGCGTAGTCTGGGACGTCGTATGGGTAATTTTCATTGA-3' (SEQ ID NO:14), respectively. An 18S rRNA-specific probe was synthesized by PCR of *S. cerevisiae* genomic DNA using sense and antisense primers having the sequences  
15 5'-AGATTAAGCCATGCATGTCT-3' (SEQ ID NO:15) and 5'-TGCTGGTACCAGACTTGCCCTCC-3' (SEQ ID NO:16), respectively. Both PCR products were individually subcloned into pCR<sup>TM</sup>II vector (Invitrogen) to generate plasmids pCR-*YCF1* and pCR-Y18S. After linearization of pCR-*YCF1* and pCR-Y18S with *Afl* and *Nco*I, a 320-nucleotide *YCF1*-specific RNA probe and 220-  
20 nucleotide 18S rRNA-specific probe were synthesized using T7 RNA polymerase and SP6 RNA polymerase, respectively. Aliquots of total RNA, prepared as described (Kohrer *et al.*, 1991, *Methods in Enzymol.* 194:390-398), from control,  $\text{CdSO}_4$ - or CDNB-pretreated DTY165 cells were hybridized with a mixture of  $^{32}\text{P}$ -labeled *YCF1* antisense probe ( $1 \times 10^6$  cpm) and 18S rRNA antisense probe ( $5 \times 10^2$  cpm) and  
25 RNase protection (Teeter *et al.*, 1990, *Mol. Cell. Biol.* 10:5728-5735) was assayed using an RPAII kit (Ambion).

#### Matrix-Assisted Laser Desorption Mass Spectrometry (MALD-MS)

The  $^{109}\text{Cd.GS}_n$  complexes purified by gel-filtration and anion-exchange chromatography were adjusted to a final concentration of 2-5 mM (as Cd)

with deionized water, mixed with an equal volume of sinapinic acid (10 mg/ml) dissolved in acetonitrile/H<sub>2</sub>O/trifluoroacetic acid (70:30:0.1 % (v/v)) and applied to the ion source of a PerSeptive Biosystems Voyager RP Biospectrometry Workstation. The instrument, which was equipped with a 1.3 m flight tube and variable two-stage ion source set at 30 kV, was operated in linear mode. Mass/charge (m/z) ratio was measured by time-of-flight after calibration with external standards.

#### Protein Estimations

Protein was estimated by a modification of the method of Peterson (1977, *Anal. Biochem.* 83:346-356).

#### Chemicals

S-(2,4-dinitrophenyl)glutathione (DNP-GS) was synthesized from 1-chloro-2,4-dinitrobenzene (CDNB) and GSH by the procedure of Kunst *et al.* (1983, *Biochem. Biophys. Acta* 983:123-125) and (Li *et al.*, 1995, *supra*). [<sup>3</sup>H]DNP-GS (specific activity, 8.7 mCi/mmol) and bimane-GS were synthesized enzymatically and purified by a modification of the procedure of Kunst *et al.* (1983, *supra*) according to Li *et al.* (1995, *supra*). Metolachlor-GS was synthesized by general base catalysis and purified by reverse-phase high performance liquid chromatography (Li *et al.*, 1995, *supra*).

GSH and CDNB were purchased from Fluka; AMP-PNP, aprotinin, ATP, creatine kinase (type I from rabbit muscle, 150-250 units/mg of protein), creatine phosphate, FCCP, oxidized glutathione (GSSG), S-methylglutathione, cysteinylglycine, cysteine, glutamate and gramicidin D, leupeptin, PMSF, verapamil, and vinblastine were from Sigma; monochlorobimane was from Molecular Probes; cellulose nitrate membranes (0.45-μm pore size, HA filters) were from Millipore; [<sup>3</sup>H]glutathione[(glycine-2-<sup>3</sup>H]-L-Glu-Cys-Gly; 44 Ci/mmol) was from DuPont NEN; and <sup>109</sup>CdSO<sub>4</sub> (78.44 Ci/mmol) was from Amersham Corp. Metolachlor was a gift from CIBA-Geigy, Greensboro, NC. All other reagents were of analytical grade and purchased from Fisher, Fluka, or Sigma.

#### Sensitivity to CDNB

If the *YCF1* gene product were to participate in the detoxification of S-conjugable xenobiotics, mutants deleted for this gene would be expected to be more sensitive to the toxic effects of these compounds than wild type cells. This is what was found (Figure 1).

5           The isogenic wild type strain DTY165 and the *ycf1*Δ mutant strain, DTY167, were indistinguishable during growth in YPD medium lacking CDNB; both strains grew at the same rate after a brief lag. However, the addition of CDNB to the culture medium caused a greater retardation of the growth of DTY167 cells (Figure 1B) than DTY165 cells (Figure 1A). Inhibitory concentrations of CDNB resulted in a  
10       slower, more linear, growth rate for at least 24 hours for both strains, but DTY167 underwent growth retardation at lower concentrations than did DTY165. The optical densities of the DTY167 cultures were diminished by 65, 82, 85, and 91% by 40, 50, 60, and 70 μM CDNB, respectively, after 24 hours of incubation (Figure 1B), whereas the corresponding diminutions for the DTY165 cultures were 14, 31, 59, and 92%  
15       (Figure 1A). The increase in sensitivity to CDNB conferred by deletion of the *YCF1* gene was similar to that seen with cadmium.

#### Impaired Vacuolar DNP-GS Transport

Vacuolar membrane vesicles purified from DTY165 cells exhibited high rates of MgATP-dependent [<sup>3</sup>H]DNP-GS uptake (Figure 2). Providing that  
20       creatine phosphate and creatine kinase were included in the uptake media to ensure ATP regeneration, addition of 3 mM MgATP increased the initial rate of DNP-GS uptake by 122-fold to a value of 12.2 nmol/mg/minute. The same membrane fraction from DTY167 cells, although capable of similar rates of MgATP-independent DNP-GS uptake, was only 17-fold stimulated by MgATP and capable of an initial rate of  
25       uptake of only 1.7 nmol/mg/minute (Figure 2).

#### Selective Impairment of Uncoupler-Insensitive Transport

Direct comparisons between vacuolar membrane vesicles from DTY165 and DTY167 cells demonstrated that deletion of the *YCF1* gene selectively abolished MgATP-energized, Δμ<sub>H</sub><sup>+</sup>-independent DNP-GS transport.

Agents that dissipate both the pH ( $\Delta\text{pH}$ ) and electrical ( $\Delta\psi$ ) components of the  $\Delta\mu_{\text{H}^+}$  established by the V-ATPase (FCCP, gramicidin D) or directly inhibit the V-ATPase, itself (bafilomycin  $\text{A}_1$ ), decreased MgATP-dependent DNP-GS uptake by vacuolar membrane vesicles from DTY165 cells from  $77.7 \pm 1.0$  nmol/mg/10 minutes to between  $43.2 \pm 1.0$  and  $47.4 \pm 1.7$  nmol/mg/10 minutes (Table 1). Ammonium chloride, which abolishes  $\Delta\text{pH}$  while leaving  $\Delta\psi$  unaffected, on the other hand, did not inhibit DNP-GS uptake (Table 1). On the basis of these characteristics, the inability of uncouplers to markedly increase the inhibitions caused by V-ATPase inhibitors, alone, and the resistance of 50-60% of total uptake to inhibition by any one of these compounds (Table 1), DNP-GS uptake by vacuolar membranes from wild type cells is concluded to proceed via two parallel mechanisms: a V-ATPase inhibitor- and uncoupler-insensitive pathway that is directly energized by MgATP, and a  $\Delta\mu_{\text{H}^+}$ -dependent, V-ATPase inhibitor-sensitive and uncoupler-sensitive pathway that is primarily driven by the inside-positive  $\Delta\psi$  established by the V-ATPase.

Of these two pathways, the  $\Delta\psi$ -dependent pathway predominated in membranes from DTY167 cells (Table 1). FCCP, gramicidin D, and bafilomycin  $\text{A}_1$  diminished net DNP-GS uptake by DTY167 vacuolar membranes from  $15.4 \pm 0.4$  nmol/mg/10 minutes to between  $4.3 \pm 0.3$  and  $6.4 \pm 0.3$  nmol/mg/10 minutes. Moreover, although the effects of FCCP or gramicidin D and V-ATPase inhibitors in combination were slightly greater than those seen when these agents were added individually, the transport remaining was only about 10% of that seen with wild type membranes and only 2-4-fold stimulated by MgATP. In conjunction with the negligible inhibitions seen with  $\text{NH}_4\text{Cl}$ , alone, indicating that  $\Delta\psi$ , not  $\Delta\text{pH}$ , is the principal driving force for the transport activity remaining in their vacuolar membranes, DTY167 cells are inferred to be preferentially impaired in MgATP-energized,  $\Delta\mu_{\text{H}^+}$ -independent DNP-GS transport.

The nonhydrolyzable ATP analog, AMP-PNP, did not promote DNP-GS uptake by vacuolar membrane vesicles from either DTY165 or DTY167 cells

(Table 1), indicating a requirement for hydrolysis of the  $\gamma$ -phosphate of ATP regardless of whether uptake was via the YCF1- or  $\Delta\psi$ -dependent pathway.

**Table 1. Effects of MgATP, MgAMP-PNP, protonophores, ionophores and V-ATPase inhibitors on [ $^3$ H]DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells.**

Uptake was measured for 10 minutes in standard uptake medium described herein containing 66.2  $\mu$ M [ $^3$ H]DNP-GS plus the compounds indicated. MgATP (3 mM) was present throughout unless otherwise indicated. MgAMP-PNP, bafilomycin A<sub>1</sub>, FCCP, gramicidin D, and NH<sub>4</sub>Cl were added at concentrations of 3 mM, 0.5  $\mu$ M, 5  $\mu$ M, 5  $\mu$ M and 1 mM, respectively. Values outside parentheses are means  $\pm$  SE ( $n = 3-6$ ); values inside parentheses are rates of uptake expressed as percentage of control.

ADDITIONS	DNP-GS UPTAKE	
	DTY165	DTY167
	(nmol/mg/10 minutes)	
Control	77.7 $\pm$ 1.0 (100)	15.4 $\pm$ 0.4 (100)
-MgATP	2.2 $\pm$ 0.4 (2.8)	1.5 $\pm$ 0.6 (9.7)
MgAMP-PNP(-MgATP)	2.5 $\pm$ 0.5 (3.2)	1.4 $\pm$ 0.3 (9.1)
FCCP	47.4 $\pm$ 1.7 (61.0)	6.4 $\pm$ 0.3 (41.8)
Gramicidin D	45.8 $\pm$ 1.4 (58.9)	5.8 $\pm$ 0.1 (37.7)
NH <sub>4</sub> Cl	69.1 $\pm$ 2.9 (88.9)	14.9 $\pm$ 0.7 (96.8)
NH <sub>4</sub> Cl + gramicidin D	42.6 $\pm$ 1.8 (54.8)	4.1 $\pm$ 0.2 (26.6)
Bafilomycin A <sub>1</sub>	43.2 $\pm$ 1.0 (55.6)	4.3 $\pm$ 0.3 (27.9)
Bafilomycin A <sub>1</sub> + gramicidin D	39.2 $\pm$ 2.6 (50.5)	3.8 $\pm$ 0.1 (24.7)

#### Abolition of High Affinity, Uncoupler-insensitive Uptake

Examination of the concentration dependence of [ $^3$ H]DNP-GS uptake revealed a near total abolition of high affinity, MgATP-dependent, uncoupler-insensitive transport by vacuolar membrane vesicles from the *ycf1* $\Delta$  mutant strain (Figure 3). When measured in the presence of uncoupler (gramicidin D), the rate of DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 cells increased as a simple hyperbolic function of MgATP (Figure 3A) and DNP-GS concentration (Figure 3B) to yield  $K_m$  values OF 86.5  $\pm$  29.5  $\mu$ M (MgATP) and 14.1  $\pm$  7.4  $\mu$ M

(DNP-GS) and a  $V_{\max}$  of  $51.0 \pm 6.3$  nmol/mg/10 minutes (DNP-GS). By contrast, uncoupler-insensitive uptake by the corresponding membrane fraction from DTY167 cells was more than 15-fold slower over the entire concentration range, showed no evidence of saturation and increased as a linear function of both DNP-GS and MgATP concentration (Figure 3).

#### Selective Inhibitors of YCF1-mediated Transport

MgATP-dependent, uncoupler-insensitive DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 cells was sensitive to inhibition by vanadate, vinblastine, verapamil, GSSG and glutathione S-conjugates other than DNP-GS (Tables 2 and 3). One hundred  $\mu$ M concentrations of metolachlor-GS, azidophenacyl-GS and biman-GS and 1 mM GSSG inhibited uptake by about 50% (Table 2), while vanadate, vinblastine, and verapamil exerted 50% inhibitions at concentrations of 179, 89 and 203  $\mu$ M, respectively (Table 3). None of these agents significantly inhibited residual MgATP-dependent, uncoupler-insensitive DNP-GS uptake by vacuolar membrane vesicles from DTY167 cells (Tables 2 and 3).

**TABLE 2. Effects of GSH, GSSG, and glutathione S-conjugates other than DNP-GS on MgATP-dependent, uncoupler-insensitive [ $^3$ H]DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells.** Uptake was measured as described for Table 1 except that 5  $\mu$ M gramicidin D was included in all of the uptake media. Values outside parentheses are means  $\pm$  SE ( $n = 3-6$ ); values inside parentheses are rates of uptake expressed as percentage of control.

COMPOUND	DNP-GS UPTAKE	
	DTY165	DTY167
	(nmol/mg/10 minutes)	
Control	47.9 $\pm$ 2.5 (100)	6.5 $\pm$ 0.8 (100)
GSH (1 mM)	50.6 $\pm$ 2.3 (105.6)	4.6 $\pm$ 1.1 (70.8)
GSSG (1 mM)	26.0 $\pm$ 0.9 (54.3)	4.4 $\pm$ 0.4 (67.7)
Metolachlor-GS (100 $\mu$ M)	27.6 $\pm$ 0.9 (57.7)	4.8 $\pm$ 0.7 (73.8)
Azidophenacyl-GS (100 $\mu$ M)	16.0 $\pm$ 1.4 (33.5)	5.2 $\pm$ 0.3 (80.0)
Bimane-GS (100 $\mu$ M)	25.2 $\pm$ 1.1 (52.6)	4.5 $\pm$ 0.4 (69.2)

**TABLE 3. Sensitivity of MgATP-dependent, uncoupler-insensitive [ $^3$ H]DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells to inhibition by vanadate, vinblastine, and verapamil.** Uptake was measured as described in Table 1 except that 5  $\mu$ M gramicidin D was included in all of the uptake media. The concentrations of the compounds causing 50% inhibition of uptake ( $I_{50}$  values) were estimated by nonlinear least squares analysis after fitting the data to a single negative exponential (Marquardt, 1963, *supra*).

Addition	$I_{50}$	
	DTY165	DTY167
	$\mu$ M	
Vanadate	179.1	Insensitive
Vinblastine	88.8	> 500
Verapamil	202.6	Insensitive

#### Vacuolar Membrane Localization

The capacity for MgATP-dependent, uncoupler-insensitive [ $^3$ H]DNP-GS uptake strictly copurified with the vacuolar membrane fraction (Table 4). By

comparison with crude microsomes (total membranes) prepared from whole spheroplast homogenates of DTY165 cells, vacuolar membrane vesicles derived from vacuoles purified by the Ficoll flotation technique were coordinately enriched for DNP-GS uptake and for both of the vacuolar membrane markers assayed,  $\alpha$ -mannosidase and bafilomycin A<sub>1</sub>-sensitive ATPase (V-ATPase) activity. The respective enrichments of MgATP-dependent, uncoupler-insensitive DNP-GS uptake,  $\alpha$ -mannosidase and bafilomycin A<sub>1</sub>-sensitive ATPase activity were 28-, 53- and 22-fold. By contrast, the vacuolar membrane fraction was 4.5-, 6.3-, 11.1- and 4.3-fold depleted of NADPH cytochrome *c* reductase (endoplasmic reticulum), latent GDPase (Golgi), vanadate-sensitive ATPase (plasma membrane), and azide-sensitive ATPase activity (mitochondrial inner membrane), respectively. Accordingly, when vacuolar membrane vesicles derived from Ficoll-flotated vacuoles were subjected to further fractionation on linear 10–40% (w/v) sucrose density gradients, MgATP-dependent, uncoupler-insensitive [<sup>3</sup>H]DNP-GS uptake,  $\alpha$ -mannosidase and bafilomycin A<sub>1</sub>-sensitive ATPase activity were found to comigrate and exhibit identical density profiles (Figure 4).



TABLE 4. Comparison of rates of MgATP-dependent, uncoupler-insensitive [ <sup>3</sup> H]DNP-GS transport and specific activities of marker enzymes in crude microsomes and vacuolar membrane vesicles prepared from DTY165 cells. Microsomes and vacuolar membrane vesicles were prepared from spheroplasts and the marker enzymes were assayed as described herein. Values shown are means ± SE (n = 3).			
PREPARATION	ACTIVITY		
	DNP-GS UPTAKE	α-mannosidase	NADPH-cyt c reductase
	nmol/mg/10 min	nmol/mg/min	nmol/mg/min
Microsomes	2.5 ± 0.3	6.3 ± 0.3	88.0 ± 1.3
Vacuolar membrane	69.9 ± 1.0	329.3 ± 3.2	19.3 ± 0.6
Enrichment (-fold)	27.96	52.27	0.22

PREPARATION	ACTIVITY			
	V-ATPase	GDPase	P-ATPase	F-ATPase
	μmol/mg/h		μmol/mg/h	
Microsomes	11.7 ± 6.3	35.0 ± 1.1	37.1 ± 4.6	155.6 ± 3.0
Vacuolar membrane	253.1 ± 15.8	5.5 ± 0.1	3.2 ± 1.6	35.1 ± 8.4
Enrichment (-fold)	21.63	0.16	0.09	0.23

#### Plasmid-encoded YCF1 Mediates Vacuolar DNP-GS Transport and

#### CDNB Resistance

Immunoblots of vacuolar membranes from pYCF1-HA-transformed DTY165 or DTY167 cells, probed with mouse anti-HA monoclonal antibody, demonstrated incorporation of YCF1-HA polypeptide into the vacuolar membrane fraction (Figure 5B). Immunoreaction with the 12CA5 epitope was not detectable in lanes loaded with membranes from pRS424-transformed cells but the same quantities of membranes prepared from pYCF1-HA-transformed cells yielded a single intensely immunoreactive band with an electrophoretic mobility ( $M_r = 156,200$ ) commensurate

with a computed mass of 172 kDa for the fusion protein encoded by YCF1-HA (Figure 5B).

5 Direct participation of the plasmid-borne YCF1-HA gene product in DNP-GS transport and CDNB detoxification was verified by the finding that vacuolar membrane vesicles purified from pYCF1-HA-transformed DTY167 cells exhibited a 6-fold enhancement of MgATP-dependent, uncoupler-insensitive [ $^3$ H]DNP-GS uptake (Figure 5A) which was accompanied by a decrease in the susceptibility of such transformants to growth retardation by exogenous CDNB (Figure 6). Whereas pYCF1-HA-transformed DTY167 cells exhibited a similar resistance to growth retardation by  
10 CDNB as untransformed DTY165 cells (compare Figure 6B with Figure 1A), the same mutant strain showed neither increased vacuolar DNP-GS transport *in vitro* nor decreased susceptibility to CDNB *in vivo* after transformation with parental plasmid pRS424, lacking the YCF1-HA insert (Figure 6B).

#### Vacuolar Accumulation of Bimane-GS *In Vivo*

15 Monochlorobimane, a membrane-permeant, nonfluorescent compound, is specifically conjugated with GSH by cytosolic glutathione *S*-transferases (GSTs) to generate the intensely fluorescent, membrane-impermeant *S*-conjugate, bimane-GS (Shrieve *et al.*, 1988, *J. Biol. Chem.* 263:14107-12114; Oude Elferink *et al.*, 1993, *Hepatology* 17:343-444; Ishikawa *et al.*, 1994, *J. Biol. Chem.* 269:29085-29093). The  
20 GS-*X* pumps of both animal and plant cells exhibit activity toward a broad range of *S*-conjugates, including bimane-GS (Ishikawa *et al.*, 1994, *supra*; Martinoia *et al.*, 1993, *supra*), and DNP-GS uptake by the yeast enzyme is shown herein to be reversibly inhibited by this compound (Table 2). These data suggest competition between bimane-GS and DNP-GS for a common uptake mechanism. Exogenous  
25 monochlorobimane therefore satisfies the minimum requirements of a sensitive probe for monitoring the intracellular transport and localization of its *S*-conjugate.

Fluorescence microscopy of DTY165 and DTY167 cells after incubation in growth medium containing monochlorobimane provides direct evidence that YCF1 contributes to the vacuolar accumulation of its glutathione *S*-conjugate by

intact cells (Figure 7). DTY165 cells exhibited an intense punctate fluorescence, corresponding to the vacuole as determined by Nomarski microscopy, after 6 hours of incubation with monochlorobimane (Figures 7A and 7C). The fluorescence associated with vacuolar bimane-GS was by comparison severely attenuated in most, and completely absent from many, DTY167 cells (Figures 7B and 7D).

*ycf1Δ* Mutants are Defective in GSH-Dependent  $\text{Cd}^{2+}$  Transport

Physiological (1 mM) concentrations of GSH (Kang, 1992, *Drug Metabolism and Disposition* 20:714-718) promoted  $\text{Cd}^{2+}$  uptake by vacuolar membrane vesicles purified from the wild type strain DTY165 but not the *ycf1Δ* mutant strain DTY167 (Figure 8). Addition of  $\text{Cd}^{2+}$  (80  $\mu\text{M}$ ) to GSH-containing media elicited MgATP-dependent, uncoupler-insensitive  $^{109}\text{Cd}^{2+}$  uptake rates of 4.5 and 0.8 nmol/mg/minute by DTY165 and DTY167 membranes, respectively (Figures 8A and 8B). Uptake by DTY165 membranes was diminished more than 9-fold by the omission of GSH (Figure 8A) whereas uptake by DTY167 membranes was slightly stimulated (Figure 8B).

GSH maximally stimulated uptake within minutes ( $t_{1/2} < 5$  minutes) of the addition of  $\text{Cd}^{2+}$  to the uptake medium and uptake was sigmoidally dependent on  $\text{Cd}^{2+}$  concentration, achieving half-maximal velocity at 120  $\mu\text{M}$  (Figure 8C).

Specific Requirement for GSH

The stimulatory action of GSH was abolished by the omission of MgATP from the assay medium (Figure 8 and Table 5) and 1 mM concentrations of GSSG, S-methylglutathione, cysteinylglycine, cysteine or glutamate did not promote MgATP-dependent, uncoupler-insensitive  $\text{Cd}^{2+}$  uptake by vacuolar membrane vesicles from either strain (Table 5).

**TABLE 5.** Effects of different GSH-related compounds on uncoupler-insensitive  $^{109}\text{Cd}$  uptake by vacuolar membrane vesicles purified from DTY165 or DTY167 cells. GSH, oxidized glutathione (GSSG), S-methylglutathione (GS-CH<sub>3</sub>), cysteinylglycine, cysteine and glutamate were added at concentrations of 1 mM. MgATP,  $^{109}\text{CdSO}_4$  and gramicidin-D were added at concentrations of 3 mM, 80  $\mu\text{M}$  and 5  $\mu\text{M}$ , respectively. Values shown are means  $\pm$  SE ( $n = 3-6$ ).

COMPOUND	$^{109}\text{Cd}$ UPTAKE (nmol/mg/10 minutes)			
	DTY165		DTY167	
	-MgATP	+MgATP	-MgATP	+MgATP
$\text{Cd}^{2+}$	5.8 $\pm$ 2.4	5.6 $\pm$ 1.5	4.3 $\pm$ 1.3	4.6 $\pm$ 2.1
$\text{Cd}^{2+}$ + GSH	4.2 $\pm$ 1.2	37.4 $\pm$ 4.5	3.3 $\pm$ 1.1	8.3 $\pm$ 2.7
$\text{Cd}^{2+}$ + GSSG	-	5.1 $\pm$ 3.2	-	3.8 $\pm$ 2.3
$\text{Cd}^{2+}$ + GS-CH <sub>3</sub>	-	4.5 $\pm$ 1.9	-	3.7 $\pm$ 3.1
$\text{Cd}^{2+}$ + Cys-Gly	-	5.6 $\pm$ 3.2	-	6.9 $\pm$ 1.4
$\text{Cd}^{2+}$ + Cys	-	7.0 $\pm$ 1.2	-	3.9 $\pm$ 1.0
$\text{Cd}^{2+}$ + Glu	-	5.7 $\pm$ 1.1	-	5.2 $\pm$ 1.3

#### Purification of Transport-Active Complex

To determine the mode of action of GSH and the form in which  $\text{Cd}^{2+}$  is transported, reaction mixtures initially containing  $\text{Cd}^{2+}$  and GSH were fractionated and YCF1-dependent uptake was assayed.

Incubation of  $^{109}\text{Cd}^{2+}$  with GSH and gel-filtration of the mixture on Sephadex G-15 yielded two major  $^{109}\text{Cd}$ -labeled peaks: a low molecular weight peak (*LMW-Cd.GS*) and a high molecular weight peak (*HMW-Cd.GS*) (Figure 9A). When rechromatographed on Mono-Q, *LMW-Cd.GS* and *HMW-Cd.GS* eluted at 0 (Figure 9C) and 275 mM NaCl, respectively (Figure 9B). Of these two  $^{109}\text{Cd}$ -labeled components, *HMW-Cd.GS* alone, underwent YCF1-dependent transport. MgATP-dependent, uncoupler-insensitive *HMW-<sup>109</sup>Cd.GS* uptake by DTY165 membranes increased as a single Michaelian function of concentration ( $K_m$ ,  $39.1 \pm 14.1 \mu\text{M}$ ;  $V_{max}$ ,  $157.2 \pm 60.7$  nmol/mg/10 minutes) (Figure 10A). By contrast, uptake of *LMW-<sup>109</sup>Cd.GS* by DTY165 membranes was negligible at all of the concentrations examined (Figure 10B). Vacuolar membranes from DTY167 cells transported neither *HMW-<sup>109</sup>Cd.GS* nor *LMW-<sup>109</sup>Cd.GS* (Figures 10A and 10B).

Bis(glutathionato)cadmium Is the Transport-Active Complex

The transport-active complex, *HMW-Cd.GS*, was identified as bis(glutathionato)cadmium ( $\text{Cd.GS}_2$ ) by three criteria: (i) The average Cd:GS molar ratio of the transported species, estimated from the  $^{109}\text{Cd}:$  $^3\text{H}$  ratios of the *HMW-Cd.GS* peaks obtained after chromatography of reaction mixtures initially containing  $^{109}\text{Cd}^{2+}$  and [ $^3\text{H}$ ]GSH on Sephadex G-15 and Mono-Q were  $0.44 \pm 0.09$  and  $0.49 \pm 0.17$ , respectively (Table 6). (ii) DTY165 membranes accumulated  $^{109}\text{Cd}$  and [ $^3\text{H}$ ]GS in a molar ratio of  $0.49 \pm 0.01$  when incubated in media containing *HMW- $^{109}\text{Cd}.$ [ $^3\text{H}$ ]GS*, MgATP and gramicidin-D (Table 6). (iii) The principal ion peak detected after MALD-MS of *HMW-Cd.GS* had an m/z ratio of  $725.4 \pm 0.7$ , consistent with the molecular weight of bis(glutathionato)cadmium (724.6 Da, Figure 11). The transport-inactive complex, *LMW-Cd.GS*, on the other hand, was tentatively identified as mono(glutathionato)cadmium on the basis of its smaller apparent molecular size (Figure 9A), failure to bind Mono-Q (Figure 9C) and Cd:GS ratio of  $0.67 \pm 0.04$  and  $0.86 \pm 0.07$  after chromatography on Sephadex G-15 and Mono-Q (Table 6), respectively.

While an m/z ratio of 725 for *HMW-Cd.GS* would be equally compatible with the transport of Cd.GSSG, this is refuted by two findings: (i) GSSG alone does not promote *YCF1*-dependent uptake (Table 5). (ii) The transport-active complex is probably a mercaptide. Pretreatment of *HMW-Cd.GS* with 2-mercaptoethanol inhibits MgATP-dependent, uncoupler-insensitive  $\text{Cd}^{2+}$  uptake by DTY165 membranes by more than 80% (Table 6) and S-methylation abolishes the stimulatory action of GSH (Table 5).

TABLE 6. Molar Cd:GS ratios of *LMW-Cd.GS* and *HMW-Cd.GS* complexes fractionated by Sephadex G-15 and Mono-Q chromatography (Figure 9) before and after MgATP-dependent, uncoupler-insensitive uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells. Cd:GS ratios were estimated from the  $^{109}\text{Cd}:[^3\text{H}]$  radioisotope ratios of samples prepared from  $^{109}\text{CdSO}_4$  and  $[^3\text{H}]\text{GSH}$ . *HMW- $^{109}\text{Cd}:[^3\text{H}]\text{GS}$*  was pretreated with 2-mercaptoethanol (2-ME) by heating a 1:4 mixture of *HMW- $^{109}\text{Cd}:[^3\text{H}]\text{GS}$*  with 2-ME at 60°C for 10 minutes before measuring  $^{109}\text{Cd}^{2+}$  uptake. Uptake was measured using 50  $\mu\text{M}$  concentrations (as Cd) of the complexes indicated in standard uptake medium containing 5  $\mu\text{M}$  gramicidin-D. Values shown are means  $\pm$  SE ( $n = 3-6$ ).

FRACTION	$^{109}\text{Cd}$ UPTAKE (nmol/mg/10 min)		MOLAR RATIO Cd:GS	
	DTY165	DTY167	BEFORE UPTAKE	AFTER UPTAKE
Sephadex G-15				
<i>HMW-Cd.GS</i>	-	-	$0.44 \pm 0.09$	-
<i>LMW-Cd.GS</i>	-	-	$0.67 \pm 0.04$	-
Mono-Q				
<i>HMW-Cd.GS</i>	$66.3 \pm 2.7$	$5.6 \pm 2.6$	$0.49 \pm 0.17$	$0.49 \pm 0.01$
<i>LMW-Cd.GS</i>	$4.4 \pm 0.8$	$3.9 \pm 1.4$	$0.86 \pm 0.07$	-
After 2-ME				
<i>HMW-Cd.GS</i>	$11.9 \pm 2.4$	$4.4 \pm 3.0$	-	-

#### Cd.GS<sub>2</sub> Transport is Directly Energized by MgATP

Purification of Cd.GS<sub>2</sub> enabled the energy requirements of YCF1-dependent transport to be examined directly and confirmed that more than 83% of the MgATP-dependent, uncoupler-insensitive Cd<sup>2+</sup> transport measured using DTY165 membranes was mediated by YCF1. Agents that dissipate both the  $\Delta\text{pH}$  and  $\Delta\Psi$  components of the H<sup>+</sup>-electrochemical gradient established by the V-ATPase (FCCP, gramicidin-D) or directly inhibit the V-ATPase, itself (bafilomycin A<sub>1</sub>), decreased MgATP-dependent Cd.GS<sub>2</sub> uptake by vacuolar membrane vesicles from DTY165 cells by 22% (Table 7). Ammonium chloride which abolishes  $\Delta\text{pH}$  while leaving  $\Delta\Psi$  unaffected, on the other hand, inhibited uptake by only 15% (Table 7). From these results and the inability of uncouplers to markedly increase the inhibitions caused by V-ATPase inhibitors alone (Table 7), Cd.GS<sub>2</sub> uptake by wild type membranes is

inferred to proceed *via* a YCF1-dependent, MgATP-energized pathway that accounts for most of the transport measured and a YCF1-independent pathway, primarily driven by the  $H^+$ -gradient established by the V-ATPase, that makes a minor contribution to total uptake.

**Table 7. Effects of uncouplers and V-ATPase inhibitors on uptake of *bis*(glutathionato)cadmium ( $Cd.GS_2$ ) by vacuolar membrane vesicles purified from DTY165 and DTY167 cells.** Uptake was measured in standard uptake medium containing 50  $\mu M$  purified  $^{109}Cd.GS_2$ . Bafilomycin  $A_1$ , FCCP, gramicidin-D and  $NH_4Cl$  were added at concentrations of 0.5  $\mu M$ , 5  $\mu M$ , 5  $\mu M$ , and 1 mM, respectively. Values outside parentheses are means  $\pm$  SE ( $n = 3-6$ ); values inside parentheses are rates of uptake expressed as percentage of control.

ADDITION	$^{109}Cd.GS_2$ UPTAKE (nmol/mg/10 minutes)	
	DTY165	DTY167
Control	105.8 $\pm$ 12.4 (100)	17.3 $\pm$ 2.7 (100)
Gramicidin-D	77.8 $\pm$ 6.4 (73.5)	9.3 $\pm$ 2.0 (56.6)
FCCP	62.2 $\pm$ 11.4 (58.8)	10.2 $\pm$ 1.6 (59.0)
$NH_4Cl$	89.8 $\pm$ 8.2 (84.8)	10.0 $\pm$ 1.7 (57.8)
$NH_4Cl$ + gramicidin-D	69.8 $\pm$ 12.0 (66.0)	8.8 $\pm$ 2.2 (50.9)
Bafilomycin $A_1$	81.8 $\pm$ 6.0 (76.6)	12.8 $\pm$ 3.6 (74.0)
Bafilomycin $A_1$ + gramicidin-D	70.2 $\pm$ 12.2 (66.4)	7.2 $\pm$ 2.4 (41.6)

#### $Cd.GS_2$ Competes with DNP-GS for Transport

As would be predicted if  $Cd.GS_2$  and the model organic GS-conjugate DNP-GS follow the same transport pathway, the  $K_i$  for inhibition of MgATP-dependent, uncoupler-insensitive  $Cd.GS_2$  uptake by DNP-GS ( $11.3 \pm 2.1 \mu M$ ; Figures 10A and 10C) coincided with the  $K_m$  for DNP-GS transport ( $14.1 \pm 7.4 \mu M$ ).

#### Pretreatment with $Cd^{2+}$ or CDNB Increases YCF1 Expression

RNase protection assays of YCF1 expression in DTY165 cells and measurements of MgATP-dependent, uncoupler-insensitive  $^{109}Cd.GS_2$  and [ $^3H$ ]DNP-GS uptake by vacuolar membranes prepared from the same cells after 24 hour of growth in media containing  $CdSO_4$  (200  $\mu M$ ) or the cytotoxic DNP-GS precursor, CDNB (150  $\mu M$ ), demonstrated a parallel increase in all three quantities. YCF1-

specific mRNA levels were increased by 1.9- and 2.5-fold by pretreatment of DTY165 cells with CdSO<sub>4</sub> and CDNB, respectively (Figure 12). The same pretreatments increased MgATP-dependent, uncoupler-insensitive <sup>109</sup>Cd.GS<sub>2</sub> uptake into vacuolar membrane vesicles by 1.4- and 1.7-fold and [<sup>3</sup>H]DNP-GS uptake by 1.6- and 2.8-fold (Figure 12).

These investigations provide the first indication of the mechanism by which *YCF1* confers Cd<sup>2+</sup> resistance in *S. cerevisiae* and its relationship to the transport of organic GS-conjugates by demonstrating that the integral membrane protein encoded by this gene specifically catalyzes the MgATP-energized uptake of bis(glutathionato)cadmium by vacuolar membrane vesicles.

The codependence of Cd-GS<sub>2</sub> and organic GS-conjugate transport on *YCF1* is evident at multiple levels: (i) The *ycf1Δ* mutant strain, DTY167, is hypersensitive to Cd<sup>2+</sup> and CDNB in the growth medium and both hypersensitivities are alleviated by transformation with plasmid-borne *YCF1*. (ii) Vacuolar membrane vesicles purified from DTY167 cells are grossly impaired for MgATP-energized, uncoupler-insensitive organic GS-conjugate and GSH-promoted Cd<sup>2+</sup> uptake. (iii) Cd.GS<sub>2</sub> and organic GS-conjugates compete for the same uptake sites on *YCF1*. (iv) Factors that increase *YCF1* expression elicit a parallel increase in Cd.GS<sub>2</sub> and organic GS-conjugate transport. Thus, a number of ostensibly disparate observations, the strong association between cellular GSH levels and Cd<sup>2+</sup> resistance (e.g., Singhal *et al.*, 1987, *FASEB J.* 1:220-223), the markedly increased sensitivity of vacuole deficient *S. cerevisiae* strains to Cd<sup>2+</sup> toxicity, and the coordinate regulation of the yeast *YCF1* and *GSH1* genes, the latter of which encodes γ-glutamylcysteine synthetase (Wemmie *et al.*, 1994, *supra*; Wu *et al.*, 1994, *supra*), are now explicable in terms of a model in which *YCF1* catalyzes the GSH-dependent vacuolar sequestration of Cd<sup>2+</sup>.

Further, at the biochemical level, *YCF1* specifically catalyzes the transport of Cd.GS<sub>2</sub> as the data provided herein establish. In addition, at the cellular level, *YCF1* confers resistance to and is induced by a spectrum of xenobiotics. Expression of *YCF1* is increased by exposure of cells to glutathione-conjugable



xenobiotics and  $\text{Cd}^{2+}$ . The close resemblance of YCF1 to MRP1, the capacity of YCF1 for both organic toxin and heavy metal transport, and its discovery in one of the most tractable and thoroughly molecularly characterized eukaryotes, *S. cerevisiae*, establishes that YCF1 is useful for manipulation of the transport of organic toxins and heavy metals in plants, mammals and yeast. Thus, according to the present invention, methods for overcoming, or at least diminishing, heavy metal contamination through bioremediation using native species or genetically engineered organisms are now possible.

#### Cloning of plant MRP1/YCF1 homologs

As described herein, the data presented herein establish that YCF1 encodes a protein functionally equivalent to human MRP1. There is next described the discovery that two plant genes, *AtMRP1* and *AtMRP2*, from *Arabidopsis* encode MRP1/YCF1 homologs.

To isolate genes likely involved in glutathione S-conjugate transport from *Arabidopsis thaliana*, degenerate PCR primers corresponding to appropriate portions of human *MRP1* (Cole *et al.*, 1992, *supra*) and *YCF1* (Szczypka *et al.*, 1994, *supra*) were designed. Four degenerate primers were synthesized but only two of these yielded amplification products of the appropriate size that hybridized with *MRP1* and *YCF1*. The sequences of the two primers were:

5'-GARAARGTIGGIATHGTIGGIMGIACIGGIGC-3'(MRP2) (SEQ ID NO:17) and 5'-TCCATDATIGTRTTIARICKTGIGC-3'(MRP4) (SEQ ID NO:18), where I = inosine, K = T or G, M = C or A and R = A or G. MRP2 corresponds to positions 1321-1331 and 1300-1310 in *MRP1* and *YCF1*, respectively; MRP4 corresponds to positions 1486-1494 and 1466-1474. Database searches confirmed that the sequences of the peptides specified by MRP2 and MRP4 were specific to *MRP1* and *YCF1* but not any other ABC transporter in GenBank database release 90 (Altschul *et al.*, 1990, *J. Mol. Biol.*, 215: 403-410).

Degenerate PCR was performed using *Arabidopsis* genomic DNA as template. Amplification was for 45 cycles using the following thermal profile: 94°C

for 30 seconds, 50°C for 30 seconds and 72°C for 1 minute. A 0.6 kb PCR product was isolated, shown to hybridize strongly with a mixed probe encompassing the second NBF domain of *MRP1* and *YCF1*, and was cloned into pCRII vector (Invitrogen).

Sequence analysis verified that the deduced translation product of the *Arabidopsis* PCR product exhibited greatest similarity to *YCF1* and *MRP1* plus an unidentified 1.7 kb *Arabidopsis* EST (ATTS1246; Hofte *et al.*, 1993, *Plant J.*, 4: 1051-1061). In order to increase the likelihood of obtaining positive clones, a mixed probe consisting of the 0.6 kb PCR product and 1.6 kb EST was employed for further screens.

Eleven independent positive clones were obtained after screening approximately  $3 \times 10^5$  plaques of a size-fractionated (3-6 kb) *Arabidopsis* cDNA library constructed in  $\lambda$ ZAPII (Kieber *et al.*, 1993, *Cell*, 72: 427-441) with the mixed probe. Restriction mapping confirmed that all 11 isolates corresponded to the same gene. The longest of these inserts, a 3.5 kb insert, designated *AtMRP2*, was subcloned and sequenced (Figure 13).

Since this isolate of *AtMRP2* was estimated to be missing approximately 1.5 kb of the 5' sequence of the ORF (assuming that the complete ORF of *AtMRP2* is similar in size to the ORFs of human *MRP1* and yeast *YCF1*), 500 bp of the most 5' sequence of *AtMRP2* was used to probe two *Arabidopsis* bacterial artificial chromosome (BAC) libraries, UCD and TAMU (Choi *et al.*, 1995, *Weeds World*, 2: 17-20) to isolate clones containing the missing sequence. This procedure yielded 8 BAC clones: U1L22, U8C12, U12A2, U23J22, U419, T9C22, T1B17 and T4K22. After digestion with *HindIII*, those fragments that hybridized with the 3.5 kb cDNA insert were introduced into pBluescript SK<sup>+</sup> and sequenced. Two of these BAC clones (T1B17, T4K22) comprise a second *MRP1* plant homolog, designated *AtMRP1*, while the remainder (U1L22, U8C12, U12A2, U23J22, U419, T9C22) comprise *AtMRP2* (see below).

After establishing that an approximately 10 kb *HindIII* fragment from BAC clone U1L22 encompassed sequences identical to *AtMRP2*, a *BglII* restriction fragment comprising the first 3 kb of the BAC clone was used to rescreen

approximately  $2 \times 10^6$  plaques from the *Arabidopsis*  $\lambda$ ZAPII cDNA library. Twenty six independent positive clones were obtained and the one containing the longest cDNA insert, 5.2 kb, was sequenced.

Sequence analysis demonstrated that the 5.2 kb cDNA was not identical to *AtMRP2* but instead a very closely related gene. Designated *AtMRP1* (Figure 16), the 5.2 kb cDNA was 84.3% and 88.2% identical to *AtMRP2* at the nucleotide and amino acid levels, respectively. Importantly, *AtMRP1* is a full-length cDNA.

Having determined the complete sequence of the *AtMRP1* cDNA, it was possible to identify the initiation codon of the *AtMRP2* genomic clone, design a specific 5'-UTR primer and amplify the remaining 5' end of *AtMRP2* to generate a full-length cDNA. Thus, full-length cDNAs encoding *AtMRP2* and *AtMRP1* (Figures 13 and 16, respectively) and genomic clones corresponding to *AtMRP2* and *AtMRP1* have been generated (Figure 14 and 17, respectively). The deduced amino acid sequences of *AtMRP2* and *AtMRP1* are presented in Figures 15 and 18, respectively.

#### 15 Expression of *AtMRP1* in *Saccharomyces cerevisiae*

The experiments described below establish that *AtMRP1* mediates the MgATP-dependent transport of GS-conjugates. The results of similar experiments on *AtMRP2* demonstrate that this gene product has the same transport capability.

The data presented herein establishes that *YCF1* from *Saccharomyces cerevisiae* encodes a 1,515 amino acid ATP-binding cassette (ABC) transporter protein which localizes to the vacuolar membrane and catalyzes MgATP-dependent GS-conjugate transport. Membrane vesicles from wild type (DTY165) cells contain two pathways for transport of the model GS-conjugate, DNP-GS: an MgATP-dependent, uncoupler-insensitive pathway and an electrically driven pathway. Membranes from the mutant strains DTY167 and DTY168, harboring a deletion of the *YCF1* gene, are by contrast more than 90% impaired in MgATP-dependent, uncoupler-insensitive DNP-GS transport. Yeast strains lacking a functional *YCF1* gene therefore represent a model system for probing the GS-conjugate transport function of plant *YCF1/MRP1* homologs.

To test the transporter capacity of *AtMRP1* (the first clone for which a full-length cDNA was obtained) for conferring GS-conjugate transport, yeast strain DTY168 (disrupted for the *YCF1* gene) was transformed with an expression vector engineered to contain the coding sequence of *AtMRP1*. After selection of the transformants, membranes were prepared and assayed for MgATP-dependent, uncoupler-insensitive DNP-GS transport as described herein. The results establish that *AtMRP1* catalyzes GS-conjugate transport in a manner indistinguishable from the vacuolar GS-X pump.

#### Construction of the expression vector

In order to constitutively express the *AtMRP1* gene in *S. cerevisiae*, a derivative of the yeast-*E. coli* shuttle vector, pYES2 (Invitrogen), was constructed. Essentially, the 831 bp *XbaI/NotI* fragment encompassing the 3-phosphoglycerate kinase (*PGK*) promoter of plasmid pFL61 (Minet et al., 1992, Plant J. 2:417-422) was inserted between the *SpeI/NotI* restriction sites of pYES2. In so doing, the galactose-inducible yeast *GAL1* promoter of pYES2 was replaced by the constitutive yeast *PGK* promoter, *pPGK*. This plasmid, designated pYES3, is otherwise identical to pYES2. The gene to be expressed is inserted into the multiple cloning site located between the *PGK* promoter and *CYC1* termination sequences.

Preliminary experiments had established that the 5' untranslated region (UTR) of the original *AtMRP1* cDNA isolate diminished expression of the open reading frame in yeast. Thus, to maximize expression, the 127 bp 5'-UTR of *AtMRP1* was removed. For this purpose, pBluescript SK<sup>-</sup>-*AtMRP1* was digested with *HpaII/SnaBI* to delete 3045 bp of the internal sequence. The remaining 5 kb fragment from this digest was gel-purified and self-ligated to generate truncated *AtMRP1* cDNA as a template for PCR. One hundred pmol of *AtMRP1*-Nco primer (5'-AAACCGGTGCGGCCCGCCATGGGGTTTGAGCCGT-3') (SEQ ID NO:19) and 100 pmol of T3 primer (5'-AATTAACCCTCACTAAAGGG-3') (SEQ ID NO:20) were used to amplify a 2002 bp fragment of *AtMRP1* using Pfu DNA polymerase

(Stratagene). Amplification was for 30 cycles using the following thermal profile: 94°C for 15 seconds; 50°C for 15 seconds; and 72°C for 3.5 minutes.

The PCR product was gel-purified, digested with *SpeI* and cloned into the *EcoRV/SpeI* sites of pBluescript SK<sup>-</sup> to generate pSK<sup>-</sup> *AtMRP1*-Nco2. The 1227 bp *SphI/SpeI* fragment of this construct was then exchanged with the 4363 bp *SphI/SpeI* fragment of pBluescript pSK<sup>-</sup> *AtMRP1* to generate pSK<sup>-</sup> *AtMRP1*-Nco5. pYES-*AtMRP1*, lacking the 5' UTR, was constructed by digesting pSK<sup>-</sup> *AtMRP1*-Nco5 with *XhoI/SpeI* to obtain a 5049 bp truncated *AtMRP1* gene fragment which was cloned into the *XhoI/XbaI* sites of pYES3. One kb of the 5' sequence of the *AtMRP1* insert of pYES3-*AtMRP1* was analyzed and was found to match exactly the sequence of the original cDNA clone.

#### Transformation of Yeast

*S. cerevisiae* strain DTY168 (MAT $\alpha$  *his6*, *leu2-3*, -112, *ura3-52 ycf1::hisG*) was transformed with pYES3-*AtMRP1* or empty vector lacking the *AtMRP1* insert (pYES3) by the LiOAc/PEG method (Giest et al., 1991, Yeast 7:253-263) and selected for uracil prototrophy by plating on AHC medium containing tryptophan (Kim et al., 1994, *supra*).

#### Isolation of membrane vesicles

For the preparation of membrane vesicles, 15 ml volumes of stationary phase cultures of the transformants were diluted into 1 L of fresh AHC medium and grown to an OD<sub>600 nm</sub> of about 1.2. Membrane vesicles were purified as described herein and in Kim *et al.* (1995, *supra*).

#### Measurement of DNP-GS uptake

DNP-GS uptake was measured as described herein in 200  $\mu$ l reaction volumes containing 3 mM ATP, 3 mM MgSO<sub>4</sub>, 5  $\mu$ M gramicidin-D, 10 mM creatine phosphate, 16 units/ml creatine kinase, 50 mM KCl, 1 mg/ml BSA, 400 mM sorbitol, 25 mM Tris-Mes (pH 8.0) and the indicated concentrations of [<sup>3</sup>H]DNP-GS (17.4 mCi/mmol). Gramicidin-D (uncoupler) was included to abolish the H<sup>+</sup>-

electrochemical potential difference that would otherwise be established by the V-ATPase in media containing MgATP.

The results of this study

5 Membrane vesicles purified from pYES3-*AtMRP1*-transformed DTY168 cells exhibit an approximately 4-fold increase in MgATP-dependent, uncoupler-insensitive [ $^3\text{H}$ ]DNP-GS uptake by comparison with membrane vesicles purified from DTY168 cells transformed with empty vector (Figure 19). When measured at a DNP-GS concentration of 61.3  $\mu\text{M}$ , the initial rates of uptake by membrane vesicles purified from pYES3-*AtMRP1*-transformed and pYES3-  
10 transformed cells were 0.4 nmol/mg/minute and 0.1 nmol/mg/minute, respectively (Figure 19).

The concentration dependence and vanadate inhibitory of uptake verify direct participation of *AtMRP1*. MgATP-dependent, uncoupler-insensitive uptake by membrane vesicles purified from the pYES3-*AtMRP1* transformants increases as a  
15 single hyperbolic function of DNP-GS concentration to yield  $K_m$  and  $V_{max}$  values of  $48.7 \pm 15.4 \mu\text{M}$  and  $6.0 \pm 1.7 \text{ nmol/mg/10 minutes}$ , respectively (Figure 19). pYES3-*AtMRP1*-dependent DNP-GS uptake decreases as a single exponential function of the concentration of the phosphoryl transition state analog vanadate, to yield an  $I_{50}$  of  $8.3 \pm 3.3 \mu\text{M}$  (Figure 20). By contrast, the apparent  $K_m$  for DNP-GS uptake by membrane  
20 vesicles purified from pYES3-transformed DTY168 cells is in excess of 500  $\mu\text{M}$  and uptake is insensitive to vanadate.

On the basis of its sequence characteristics and the results of these experiments, *AtMRP1* encodes the vacuolar GS-X pump. The increases in uptake following the introduction of plasmid borne *AtMRP1* into yeast (*ca.* 4 nmol/mg/20  
25 minutes) are commensurate with the rates of MgATP-dependent, uncoupler-insensitive DNP-GS uptake measured in vacuolar membrane vesicles purified from plant sources (2.3, 3.8, 18.2, 5.8, and 2.1 nmol/mg/20 minutes for *Arabidopsis* leaf, *Arabidopsis* root, *Beta vulgaris* storage root, *Vigna radiata* hypocotyl and *Zea mays* root, respectively) (Table III in Li *et al.*, 1995, *supra*). The  $K_m$  for DNP-GS transport by heterologously

expressed AtMRP1 is similar to that reported for the endogenous GS-X pump of plant vacuolar membranes ( $81.3 \pm 41.8 \mu\text{M}$ , Li *et al.*, 1995, *supra*). The  $I_{50}$  for inhibition of AtMRP1-dependent DNP-GS transport by vanadate coincides with the  $I_{50}$  for inhibition of the endogenous vacuolar GS-X pump of plant cells ( $7.5 \pm 3.9 \mu\text{M}$ , Li *et al.*, 1995, *supra*).

Having confirmed that the endogenous vacuolar GS-X pump of *S. cerevisiae* is lacking in the *ycf1*  $\Delta$  mutant strains, DTY168 and DTY167 (Li *et al.*, 1995, *supra*), and in any case has a markedly lower  $K_m$  for DNP-GS and is 6 to 8-fold less sensitive to vanadate than the plant cognate, these findings establish that AtMRP1 *per se* is responsible for the MgATP-dependent, uncoupler-insensitive transport measured in these experiments. Given that heterologous expression of *AtMRP1* alone is sufficient for DNP-GS transport in DTY168 cells, it is concluded that one of the GS-X pumps of *Arabidopsis* has been cloned in its entirety.

Sequence comparisons of MRP1, cMOAT, YCF1, AtMRP1 and AtMRP2 with other members of the ABC transporter superfamily reveal two major subgroups. One group contains MRP1, cMOAT, YCF1, AtMRP1, AtMRP2 and the *Leishmania* P-glycoprotein-related molecule (Lei/PgpA). The other group contains the MDRs, the major histocompatibility complex transporters and STE6. However, of all the ABC transporters defined to date, cMOAT, YCF1, AtMRP1 and AtMRP2 exhibit the closest resemblance to each other. Unlike the similarities between the GS-X pump subgroup, Lei/PgpA and CFTR, which center on the nucleotide binding folds (NBFs), the similarities between the GS-X pump members cMOAT, YCF1, AtMRP1 and AtMRP2 are found throughout the sequence. GS-X family members are 40-45% identical (60-65% similar) at the amino acid level, possess NBFs with an equivalent spacing of conserved residues and are colinear with respect to the location, extent and alteration of putative transmembrane spans and extramembrane domains. Two features of members of the GS-X pump family that distinguish them from other ABC transporters are their possession of a central truncated CFTR-like regulatory domain

rich in charged amino acid residues and an approximately 200 amino acid residue N-terminal extension.

A hydropathy alignment of AtMRP1, AtMRP2, YCF1, HmMRP1, and RtCMOAT is shown in Figure 21. Note the following: (i) The almost exact equivalence of AtMRP1 and AtMRP2 with respect to the alternation of hydrophobic and hydrophilic stretches. (ii) The close correspondence of AtMRP1 and AtMRP2 with all of the other members of the MRP1/YCF1/cMOAT subclass of ABC transporters in terms of the overall hydropathy profiles. (iii) The "signature" profile for the N-terminal 200 amino acid residues of all of the sequences shown, which is unique to the MRP1/YCF1/cMOAT subclass. Hydropathy was computed according to Kyte and Doolittle (1982, *J. Mol. Biol.* 46:105-132) over a running window of 15 amino acid residues. Hydrophobic stretches of sequence fall below the line and hydrophilic stretches fall above the line.

In Figure 22 there is depicted domain comparisons between AtMRP1, ScYCF1, HmMRP1, RtCMOAT, RbEBCR and HmCFTR. The domains indicated are the N-terminal extension (NH<sub>2</sub>), first and second transmembrane spans (TM1 and TM2, respectively), first and second nucleotide binding folds (NBF1 and NBF2, respectively), putative CFTR-like regulatory domain (R), and the C-terminus (COOH). This comparison is also tabulated in Tables 8 and 9.

**TABLE 8: Identity and similarity analysis of putative domains of AtMRP1 against AtMRP2 ScYCF1, HmMRP1, RtCMOAT, HmCFTR and RbEBCR. ScYCF1, *Saccharomyces cerevisiae* YCF1; HmMRP1, human MRP1; RtCMOAT, rat cMOAT; HmCFTR, human CFTR; RbEBCR, rabbit EBCR. The domains identified are N-terminal extension (NH<sub>2</sub>), transmembrane segments 1 and 2 (TM1 and TM2, respectively), CFTR-like regulatory domain (R), nucleotide binding folds 1 and 2 (NBF1 and NBF2, respectively) and C-terminus (COOH). Similarity was calculated as described herein over the sequence segments indicated in Table 9.**

SEQUENCE	DOMAIN	OVERALL	NH <sub>2</sub>	TM1	NBF1
AtMRP2	Identity	87.0	74.4	90.4	92.1
	Similarity	93.7	85.2	96.1	96.7
ScYCF1	Identity	36.1	13.3	32.2	50.0



SEQUENCE	DOMAIN	OVERALL	NH <sub>2</sub>	TM1	NBF1
	<i>Similarity</i>	55.4	32.9	52.6	75.0
HmMRP1	<i>Identity</i>	41.5	16.2	37.4	58.0
	<i>Similarity</i>	63.3	34.8	57.5	78.7
RtCMOAT	<i>Identity</i>	38.6	19.6	33.8	58.7
	<i>Similarity</i>	60.2	36.7	61.0	80.0
HmCFTR	<i>Identity</i>	29.2	0	22.8	40.7
	<i>Similarity</i>	55.1	0	47.8	62.0
RbEBCR	<i>Identity</i>	38.9	17.2	34.5	62.4
	<i>Similarity</i>	60.4	34.9	61.6	82.6
SEQUENCE	DOMAIN	R	TM2	NBF2	COOH
AtMRP2	<i>Identity</i>	80.5	86.9	91.3	89.4
	<i>Similarity</i>	89.8	94.2	96.5	94.4
ScYCF1	<i>Identity</i>	33.9	34.7	34.7	58.1
	<i>Similarity</i>	59.5	57.9	57.9	71.8
HmMRP1	<i>Identity</i>	31.6	31.9	61.9	48.3
	<i>Similarity</i>	50.4	56.3	72.8	69.0
RtCMOAT	<i>Identity</i>	33.9	34.4	60.7	50.0
	<i>Similarity</i>	50.0	58.8	75.7	67.2
HmCFTR	<i>Identity</i>	45.7	22.3	39.5	28.1
	<i>Similarity</i>	75.0	51.3	61.6	58.4
RbEBCR	<i>Identity</i>	35.6	34.1	61.9	43.0
	<i>Similarity</i>	51.7	59.1	74.0	62.7

TABLE 9. Positions and sizes of segments of sequence analyzed in Table 8.					
SEQUENCE	DOMAIN	OVERALL	NH <sub>2</sub>	TM1	NBF1
AtMRP1	<i>Position</i>		1-223	224-631	634-782
	<i>Size</i>	1622	223	407	148
AtMRP2	<i>Position</i>		1-223	224-631	634-782

SEQUENCE	DOMAIN	OVERALL	NH <sub>2</sub>	TM1	NBF1
	Size	1622	223	407	148
ScYCF1	Position		1-210	211-645	646-787
	Size	1515	210	435	142
HmMRP1	Position		1-240	241-660	661-810
	Size	1531	240	420	150
RtCMOAT	Position		1-192	193-648	649-799
	Size	1540	192	456	151
HmCFTR	Position		0	1-440	441-590
	Size	1481	0	440	150
RbEBCR	Position		1-193	194-651	652-800
	Size	1562	193	458	149
SEQUENCE	DOMAIN	R	TM2	NBF2	COOH
AtMRP1	Position	783-900	901-1244	1245-1417	1418-1622
	Size	117	343	172	205
AtMRP2	Position	783-905	906-1249	1250-1422	1423-1622
	Size	122	343	172	200
ScYCF1	Position	788-936	937-1279	1280-1453	1454-1515
	Size	149	343	174	163
HmMRP1	Position	811-960	961-1300	1301-1473	1474-1531
	Size	150	340	173	59
RtCMOAT	Position	800-960	961-1302	1303-1476	1477-1541
	Size	161	342	174	65
HmCFTR	Position	591-847	848-1217	1218-1389	1390-1481
	Size	256	371	172	92
RbEBCR	Position	801-961	962-1304	1305-1477	1478-1562
	Size	161	343	173	86

As is apparent from the data presented above, there is significant homology between similar domains among AtMRP-related proteins. In particular, the N-terminal and R domains share significant homology among the AtMRP-related proteins tested. These data establish that in addition to primary sequence, the secondary structure of the molecule plays a significant role in GS-X pump function.

It should be appreciated that *AtMRP1* and *AtMRP2* constitute a family of genes in *Arabidopsis*, wherein various members of the family have different substrate specificities as demonstrated by the next set of experiments.

#### Substrate Preferences of AtMRP1 and AtMRP2

To examine the substrate preferences of AtMRP1 and AtMRP2, the following experiments were performed.

#### Isolation of Bn-NCC-1

[<sup>14</sup>C]Bn-NCC-1 (33.3 mCi/mmol) was extracted from senescent cotyledons of rape (*Brassica napus*) and was purified by preparative HPLC (Krautler *et al.*, 1992, *Plant Physiol. Biochem.* 30:333-346). Determination of the purity of the final preparation by analytical HPLC and enumeration of concentration and specific radioactivity (33.3 mCi/mmol) were performed according to Hinder *et al.* (1966, *J. Biol. Chem.* 271:27233-27236). Unlabeled Bn-NCC-1 was isolated from fully senescent cotyledons of excised shoots that had been maintained in complete darkness for 1 week.

#### Measurement of transport

Cells were grown and vacuolar membrane-enriched vesicles were prepared as described (Kim *et al.*, 1995, *J. Biol. Chem.* 270:2630-2635-). Uptake of [<sup>14</sup>C]Bn-NCC-1, [<sup>3</sup>H]C3G-GS, [<sup>3</sup>H]DNP-GS, [<sup>3</sup>H]GSSG, [<sup>14</sup>C]metolachlor or [<sup>3</sup>H]taurocholate was measured routinely in 200  $\mu$ l reaction volumes containing membrane vesicles (10-20  $\mu$ g protein), 3 mM ATP, 3 mM MgSO<sub>4</sub>, 5  $\mu$ M gramicidin-D, 10 mM creatine phosphate, 16 units/ml creatine phosphate kinase, 50 mM KCl, 1mg/ml BSA, 400 mM sorbitol, 25 mM Tris-Mes (pH 8.0) and the indicated

concentrations of transport substrate. Uptake was terminated by the addition of 1 ml ice-cold wash medium (400 mM sorbitol/3 mM Tris-Mes, pH 8.0) and vacuum filtration of the suspension through prewetted Millipore HA cellulose nitrate filters (pore size 0.45  $\mu$ m). The filters were rinsed twice with wash medium, and the retained radioactivity was determined by liquid scintillation counting. Nonenergized uptake was estimated by the same procedure except that ATP was omitted from the uptake medium.

The effect of taurocholate on the release of [ $^3$ H]DNP-GS from membrane vesicles that had been allowed to mediate AtMRP2-dependent accumulation of this compound during a preceding uptake period was determined. This was accomplished by rapid depletion of ATP from the uptake medium using a hexokinase trap (glucose + ATP  $\rightarrow$  glucose-6-phosphate + ADP) and measurements of the decrease in vesicular radiolabel in the presence or absence of taurocholate. Membranes from DTY168/pYES3-AtMRP2 cells were incubated for 10 minutes in standard uptake medium containing 61.3  $\mu$ M [ $^3$ H]DNP-GS after which time 200 mM glucose and 50 units/ml hexokinase (Type F-300 from baker's yeast) were added. After incubation for a further 2 minutes, taurocholate (50  $\mu$ M) or Triton X-100 (9.01% v/v) was added and release of vesicular [ $^3$ H]DNP-GS was measured as described. Control samples were treated identically except that no additions were made after the initial 10 minute incubation period.

#### Substrate Preferences

The absence of AtMRP2-dependent transport from DTY168 and DTY168/pYES3 membranes and the selective inhibition of this system by micromolar concentrations of vanadate, established that AtMRP2-dependent transport may be measured in two ways. This may be accomplished by assessing the difference between the rates of MgATP-dependent, uncoupler-insensitive uptake by DTY168/pYES3-AtMRP2 membranes by comparison with DTY168 or DTY168/pYES3 membranes, or by assessing the vanadate-sensitive component of MgATP-dependent, uncoupler-insensitive uptake by DTY168/pYES3-AtMRP2 membranes. Because the results were

qualitatively and quantitatively similar whichever method was used, "AtMRP2-dependent" transport as used in this section refers to uptake which is measured as the increment consequent on transformation of DTY168 cells with pYES3-AtMRP2 *versus* pYES3.

5 Application of this methodology to vacuolar membrane-enriched vesicles purified from pYES3-AtMRP2- *versus* pYES3-transformed DTY168 cells and expansion of the transport assays to measurements of the concentration dependence of [<sup>3</sup>H]DNP-GS, [<sup>3</sup>H]GSSG, [<sup>14</sup>C]metolachlor-GS, [<sup>14</sup>C]Bn-NCC-1 and [<sup>3</sup>H]taurocholate uptake, demonstrated that the substrate preferences and maximal transport capacities of  
10 AtMRP2 and AtMRP1 differed markedly. While uptake of all of the GSH derivatives examined conformed to Michaelis-Menten kinetics, the  $V_{\max}$  values for AtMRP2-dependent uptake were consistently severalfold greater than those for AtMRP1-dependent uptake. The  $V_{\max}$  values for AtMRP2-dependent uptake of [<sup>3</sup>H]DNP-GS, [<sup>3</sup>H]GSSG and [<sup>14</sup>C]metolachlor-GS were  $16.3 \pm 3.1$ ,  $38.1 \pm 3.2$  and  $136.0 \pm 28.1$   
15 nmol/mg/10 min, respectively; the corresponding values for AtMRP1 were  $8.2 \pm 1.6$ ,  $6.8 \pm 1.1$  and  $17.5 \pm 5.2$  nmol/mg/10 min. With the exception of [<sup>3</sup>H]GSSG whose  $K_m$  for AtMRP1-dependent uptake ( $21.9.2 \pm 58.3 \mu\text{M}$ ) was three times greater than that for AtMRP2-dependent uptake ( $73.0 \pm 15.1 \mu\text{M}$ ), the  $K_m$  values estimated for AtMRP2 and AtMRP1 were very similar ( $65.7 \pm 29.8$  *versus*  $63.6 \pm 36.5 \mu\text{M}$  for metolachlor-GS).

20 Single concentration ( $50 \mu\text{M}$ ) measurements of uptake of the glutathionated anthocyanin, cyanin-3-glucoside-GS (C3G-GS), demonstrated an approximately 6-fold greater capacity of AtMRP2 for transport of this compound (rate =  $48.4 \pm 2.2$  nmol/mg/10 min) by comparison with AtMRP1 (rate =  $7.9 \pm 0.7$  nmol/mg/10min).

25 In no case was MgATP-dependent, uncoupler-insensitive uptake of the unconjugated precursors of the GS-compounds, DNP, GSH, metolachlor and C3G detectable.

Neither AtMRP2 nor AtMRP1 catalyzed the uptake of [<sup>3</sup>H]taurocholate. Transformation of DTY168 cells with either pYES3-AtMRP2 or pYES3-AtMRP1

conferred little or no increase in the capacity of vacuolar membrane-enriched vesicles for [ $^3\text{H}$ ]taurocholate uptake over that measured with vesicles prepared from pYES3-transformed cells. The results of these experiments are presented in Table 10.

Table 10. Kinetic parameters for uncoupler-insensitive AtMRP1- and AtMRP2-dependent transport of GS-derivatives, *Bn*-NCC-1 and taurocholate.

Compound	AtMRP1		AtMRP2	
	$K_m$	$V_{max}$	$K_m$	$V_{max}$
DNP-GS	$73.8 \pm 18.8$	$8.2 \pm 1.6$	$65.7 \pm 29.8$	$16.3 \pm 3.1$
GSSG	$219.2 \pm 58.3$	$6.8 \pm 1.1$	$73.0 \pm 15.1$	$38.1 \pm 3.2$
Metolachlor-GS	$63.6 \pm 36.5$	$17.5 \pm 5.2$	$75.1 \pm 31.6$	$136.0 \pm 28.1$
<i>Bn</i> -NCC-1	-----Linear-----		$15.2 \pm 2.3$	$63.1 \pm 2.5$
Taurocholate	-----Linear-----		-----Linear-----	

MgATP-dependent, uncoupler-insensitive uptake by DTY168/pYES3-AtMRP1, DTY168/pYES3-AtMRP2 and DTY168/pYES3 membranes was measured as described herein. The  $K_m$  and  $V_{max}$  values were estimated by fitting the data to a single Michaelis-Menten function by nonlinear least squares analysis. Values shown are means  $\pm$  SE.

The 2- to 8-fold greater capacity of AtMRP2 *versus* AtMRP1 for transport of the compounds examined was not attributable to differences in the levels of expression of their cDNAs from the *PGK* gene promoter of pYES3. Quantitative RT-PCR of equivalent amounts of total RNA extracted from DTY168/pYES3-AtMRP2 and DTY168/pYES3-AtMRP1 cells yielded similar levels of the 800 bp PCR

amplification product predicted from the sequences of the oligonucleotide primers used. Since neither amplification product was generated when PCR was performed without reverse transcription or when total RNA from DTY168/pYES3 cells was employed as template, contamination by genomic DNA and/or RT-PCR of transcripts other than those from *AtMRP2* or *AtMRP1*, respectively, was not responsible for the observed results.

#### Anomalous interactions between candidate transport substrates

Two critical properties of *AtMRP2* were its capacity for the simultaneous transport of GS-conjugates and *Bn*-NCC-1 and its pronounced sensitivity to inhibition by taurocholate. Simultaneous measurements of [ $^{14}\text{C}$ ]*Bn*-NCC-1 and [ $^3\text{H}$ ]DNP-GS uptake by membrane vesicles purified from DTY168/pYES3-*AtMRP2* cells revealed parallel accumulation of both compounds with little or no interference of the transport of one by the other. *AtMRP2*-dependent uptake of [ $^{14}\text{C}$ ]*Bn*-NCC-1 at an extravesicular concentration equivalent to its  $K_m$  value ( $15\mu\text{M}$ ) was nearly three times less sensitive to DNP-GS than would be predicted if this GS-conjugate were a competitor. If DNP-GS were a simple competitive inhibitor such that its  $K_m$  value ( $66\mu\text{M}$ ) approximated its  $K_i$  value for the inhibition of *Bn*-NCC-1 uptake,  $120\mu\text{M}$  DNP-GS would be expected to inhibit [ $^{14}\text{C}$ ]*Bn*-NCC-1 uptake by 48% but this was not observed. DNP-GS concentrations in excess of  $120\mu\text{M}$  decreased [ $^{14}\text{C}$ ]*Bn*-NCC-1 uptake by less than 18%. Reciprocally, the concentration-dependence of *AtMRP2*-mediated [ $^3\text{H}$ ]DNP-GS uptake was not affected appreciably by *Bn*-NCC-1. The  $K_m$  and  $V_{\max}$  values for *AtMRP2*-dependent [ $^3\text{H}$ ]DNP-GS uptake in the presence of  $15\mu\text{M}$  *Bn*-NCC-1 ( $80.5 \pm 28.6\mu\text{M}$  and  $18.3 \pm 1.6\text{ nmol/mg/10/min}$ ) were similar to those measured in its absence.

Although neither *AtMRP2* nor *AtMRP1* transported taurocholate, *AtMRP2*-mediated transport was selectively inhibited by this compound. *AtMRP1*-dependent [ $^3\text{H}$ ]DNP-GS uptake was relatively insensitive to taurocholate ( $I_{50} > 250\mu\text{M}$ ) but the uptake of both [ $^3\text{H}$ ]DNP-GS and [ $^{14}\text{C}$ ]*Bn*-NCC-1 by *AtMRP2* was strongly inhibited ( $I_{50(\text{DNP-GS uptake})} = 27 \pm 1.3\mu\text{M}$ ;  $I_{50(\text{Bn-NCC-1 uptake})} = 49.5 \pm 0.3\mu\text{M}$ ).

Taurocholate at the concentrations employed appeared to exert its effect on AtMRP2-mediated transport by inhibiting pump activity directly rather than by increasing background membrane permeability and decreasing net influx by increasing passive DNP-GS efflux. Addition of taurocholate at a concentration (50  $\mu$ M) sufficient to inhibit AtMRP2-dependent [ $^3$ H]DNP-GS uptake by 70% to DTY168/pYES3-AtMRP2 vesicles that had accumulated [ $^3$ H]DNP-GS for 10 minutes before arresting pump action by ATP depletion using a hexokinase trap, did not accelerate the efflux of intravesicular  $^3$ H-label over that measured on vesicles subject to a hexokinase trap in the absence of taurocholate. Imposition of a hexokinase trap and addition of a concentration of detergent (Triton X-100; 0.01% v/v) known to permeate these membranes (Zhen *et al.*, 1997, *J. Biol. Chem.* 272:22340-22348), on the other hand, increased the rate and extent of release of the [ $^3$ H]DNP-GS accumulated during the preceding 10 minute uptake period by more than 3-fold *versus* DTY168/pYES3-AtMRP2 vesicles treated with hexokinase alone or hexokinase plus taurocholate.

The high capacity of AtMRP2 for the transport of large amphipathic anions other than GS-conjugates (i.e., *Bn*-NCC demonstrates that one pump can assume more than one of the several ABC transporter-like functions identified in plants to date. In the case of AtMRP2, this includes transport activity directed to a broad-range GS-conjugate pump and a chlorophyll metabolite pump. Thus, on the one hand, the high capacity of heterologously-expressed AtMRP2, and to a lesser extent AtMRP1, for the transport of metolachlor-GS, and by extension GS-conjugates of other herbicides to glutathionation, is consistent with the molecular identification of transporters capable of removing these and related compounds from the cytosol. On the other hand, the high capacity of AtMRP2 for the transport of *Bn*-NCC is consistent with the identification of an element capable of contributing to the further metabolism and eventual removal of tetrapyrrole derivatives generated during leaf senescence from the cytosol.

Vacuolar uptake of glutathionated medicarpin by the glutathione conjugate pump



A key event in the disease resistance response of legumes is the rapid and localized accumulation of isoflavonoid phytoalexins. Accordingly, most studies of plant-pathogen interactions in the Leguminosae have centered on the enzymology and molecular biology of the isoflavonoid biosynthetic pathway (Dixon *et al.*, 1995, *Physiol. Plant* 93:385). However, the mechanism and sites of intracellular accumulation of these compounds is not understood. Since many isoflavonoid phytoalexins are as toxic to the host plant as they are to its pathogens, it is essential that they are accumulated in the plant in a site which is sequestered (*i.e.*, isolated) from the cytoplasm.

The following experiments describe uptake of free [ $^3\text{H}$ ]medicarpin by vacuolar membrane vesicles purified from etiolated hypocotyls of mung bean (*Vigna radiata*). This uptake is slow and relatively insensitive to MgATP. However, after incubation with glutathione and a total glutathione-S-transferase preparation from maize (*Zea mays*), [ $^3\text{H}$ ]medicarpin uptake occurs at a rate which is 8-fold faster in the presence, as opposed to the absence of MgATP. MgATP-dependent uptake of glutathione/glutathione-S-transferase pretreated [ $^3\text{H}$ ]medicarpin is only slightly inhibited by uncoupler (gramicidin D), but is strongly inhibited by vanadate and the model glutathione-S-conjugate, S-(2,4-dinitrophenyl)glutathione. These results demonstrate that the MgATP-energized glutathione-conjugate pump identified herein in the membrane preparation is capable of high affinity, high capacity transport of glutathionated isoflavonoid phytoalexins. The experimental procedures and results of these experiments are now described.

#### Preparation of [ $^3\text{H}$ ]medicarpin

[ $^3\text{H}$ ]medicarpin was produced by base-catalyzed tritium exchange from  $^3\text{H}_2\text{O}$  using unlabeled medicarpin isolated from fenugreek (*Trigonella foenumgraecum*) seedlings exposed to 3 mM  $\text{CuCl}_2$ .

### GST purification and conjugation of medicarpin

Two-week old maize (*Zea mays*) B73N seedlings were grown under continuous light at 21°C. Twenty four hours prior to harvesting, the seedlings were exposed to a mild treatment with 2,4-dichlorophenoxyacetic acid and atrazine to stimulate GST expression (Timmerman, 1989, *Physiol. Plant* 77:465). Two-gram samples of root and shoot tissue were ground to homogeneity in 50 ml of 500 mM sodium phosphate buffer, pH 7.8 (Buffer A). The extract was centrifuged at 7,000 x g for 10 minutes at 4°C and the resulting supernatant was filtered through Miracloth and mixed with 2 ml of S-hexylglutathione-conjugated agarose beads (Sigma). After incubation for 5 minutes at 21°C, the beads were sedimented by centrifugation at 500 x g at 4°C. The supernatant was discarded and the beads were resuspended in 2.5 ml of prechilled Buffer A and centrifuged again. Bound GST was eluted by resuspension of the beads in 2 ml Buffer B (20 mM GSH, 500 mM sodium phosphate, pH 7.8) and incubation for 5 minutes at 21°C. The beads were sedimented by centrifugation at 500 x g and the supernatant was assayed for GST activity (Mannervick *et al.*, 1981, *Methods Enzymol.*, 77:231).

[<sup>3</sup>H]medicarpin (0.5 µCi, 4.5 Ci/mol) was conjugated with GSH by incubation with 25 µl of total purified maize GSTs for 3 hours at 21°C in the dark. Control, unconjugated samples were prepared by mixing [<sup>3</sup>H]medicarpin (0.5 µCi) with cold Buffer B and immediately freezing the mixture in liquid nitrogen.

### Synthesis of S-(2,4-dinitrophenyl)glutathione (DNP-GS)

DNP-GS was synthesized from 1-chloro-2,4-dinitrobenzene and GSH by a modification of the enzymatic procedure of Kunst *et al.*, (1989, *Biochim. Biophys. Acta* 983:123; Li *et al.*, 1995, *supra*).

### Preparation of vacuolar membrane vesicles

Vacuolar membrane vesicles were purified from etiolated hypocotyls of *V. radiata* cv. Berken as described (Li *et al.*, *Plant Physiol.* 109: 1257, Li *et al.*, 1995, *supra*).

### Measurement of uptake

Unless otherwise indicated, [ $^3\text{H}$ ]medicarpin or [ $^3\text{H}$ ]medicarpin-GS uptake was measured at 25°C in 200  $\mu\text{l}$  reaction volumes containing 3 mM ATP, 3 mM  $\text{MgSO}_4$ , 10 mM creatine phosphate, 16 U/ml creatine kinase, 50 mM KCl, 0.1% (w/v) BSA, 400 mM sorbitol and 25 mM Tris-Mes buffer, pH 8.0. Uptake was initiated by the addition of 12  $\mu\text{l}$  membrane vesicles (30-40  $\mu\text{g}$  protein) and brief mixing of the samples on a vortex mixer. Uptake was terminated by the addition of 1 ml ice-cold wash medium (400 mM sorbitol, 3 mM Tris-Mes, pH 8.0) and vacuum filtration of the suspension through prewetted HA cellulose nitrate filters (pore diameter 0.45  $\mu\text{m}$ ). The filters were rinsed twice with a 1 ml ice-cold wash medium, air-dried and radioactivity was determined by liquid scintillation counting.

Protein estimations and source of commercial chemicals was as described herein.

### Results

Appreciable MgATP-dependent uptake of [ $^3\text{H}$ ]medicarpin by vacuolar membrane vesicles purified from etiolated hypocotyls of mung was dependent on preincubation of this compound with GSH and GSTs. Free [ $^3\text{H}$ ]medicarpin incubated in the presence of GSH in the absence of GSTs was taken up at  $16.7 \pm 3.6$  and  $7.4 \pm 1.3$  nmol/mg/20 minutes in the presence and absence of MgATP, respectively (Figure 25). In contrast, [ $^3\text{H}$ ]medicarpin-GS synthesized by incubating [ $^3\text{H}$ ]medicarpin with GSH in the presence of affinity-purified maize GSTs, was taken up at  $81.0 \pm 13.3$  and  $11.3 \pm 0.4$  nmol/mg/20 minutes in the presence and absence of MgATP, respectively (Figure 25).

MgATP-dependent [ $^3\text{H}$ ]medicarpin-GS uptake was strongly inhibited by vanadate and DNP-GS but was relatively insensitive to uncouplers. Whereas inclusion of vanadate (10  $\mu\text{M}$ ) or DNP-GS (100  $\mu\text{M}$ ) in the assay medium inhibited [ $^3\text{H}$ ]medicarpin uptake by more than 85%, addition of the ionophore, gramicidin D, diminished uptake by only 17% (Table 11).

Table 11. Effects of different inhibitors on [ <sup>3</sup> H]medicarpin-GS uptake by vacuolar membrane vesicles. [ <sup>3</sup> H]medicarpin-GS was added at a concentration of 65 $\mu$ M. MgATP was either omitted (-MgATP) or added at a concentration of 3 mM (+MgATP). Gramicidin-D, vanadate and DNP-GS were added at concentrations of 5 $\mu$ M, 10 $\mu$ M and 100 $\mu$ M, respectively. Values outside parentheses are means $\pm$ SE ( $n = 3$ ); values inside parentheses are rates of uptake expressed as percentage of control.		
TREATMENT	[ <sup>3</sup> H]Medicarpin-GS Uptake (nmol/mg/10 minutes)	
	+MgATP	-MgATP
Control	85.6 $\pm$ 13.3 (100)	16.7 $\pm$ 6.0 (100)
+ Gramicidin-D	71.2 $\pm$ 3.0 (83.2)	13.2 $\pm$ 2.1 (79.0)
+ Gramicidin-D + vanadate	12.9 $\pm$ 0.9 (15.1)	17.4 $\pm$ 0.5 (104.2)
+ Gramicidin-D + DNP-GS	11.7 $\pm$ 2.8 (13.7)	5.6 $\pm$ 3.1 (33.5)

MgATP-dependent, uncoupler-insensitive uptake increases as a single Michaelian function of [<sup>3</sup>H]medicarpin-GS concentration to yield  $K_m$  and  $V_{max}$  values of  $21.5 \pm 15.5 \mu\text{M}$  and  $77.8 \pm 23.3 \text{ nmol/mg/20 minutes}$ , respectively (Figure 26).

Direct involvement of the GS-X pump in the accumulation of [<sup>3</sup>H]medicarpin-GS by vacuolar membrane vesicles is therefore evident at three levels: (i) Glutathionation of medicarpin selectively increases MgATP-dependent uptake. MgATP-independent uptake is marginally affected by glutathionation but MgATP-dependent uptake is stimulated by approximately six-fold confirming that medicarpin-GS is the transported species and MgATP is the energy source. (ii) Uptake is directly energized by MgATP. The inability of uncoupler to markedly inhibit [<sup>3</sup>H]medicarpin-GS uptake implies that the  $\text{H}^+$ -electrochemical gradient that would otherwise be established by the vacuolar  $\text{H}^+$ -ATPase in the presence of MgATP does not drive uptake. Rather, the pronounced inhibition of MgATP-dependent uptake exerted by vanadate agrees with the notion that GS-X-mediated uptake is strictly dependent on ATP hydrolysis and formation of a phosphoenzyme intermediate (Martinoia *et al.* 1993, *supra*; Li *et al.*, 1995, *supra*), (iii) [<sup>3</sup>H]medicarpin-GS and the model GS-X pump substrate DNP-GS, whose transport has been exhaustively analyzed in this system as

described herein, compete for uptake indicating that both are transported by the same moiety.

5 The efficacy of medicarpin-GS as a substrate for the vacuolar GS-X pump is striking. Even though the  $K_m$  for medicarpin-GS uptake is undoubtedly an overestimate, since the yield from the conjugation reaction was not enumerated, it is nevertheless 2 to 25-fold lower than those estimated previously for DNP-GS, C3G-GS (80 and 46  $\mu\text{M}$  in this system), glutathione-S-N-ethylmaleimide (500  $\mu\text{M}$ ) and metolachlor-GS (40-60  $\mu\text{M}$ , barley vacuoles; Martinoia *et al.*, 1993, *supra*). Moreover, the capacity of the GS-X pump for medicarpin-GS uptake is high ( $V_{\text{max}} = 78$  10 nmol/mg/20 minutes) versus DNP-GS ( $V_{\text{max}} = 12$  nmol/mg/20 minutes) and comparable to that estimated for C3G-GS ( $V_{\text{max}} = 45$  nmol/mg/minute). Thus, while maize anthocyanin was the first natural substrate shown to be vacuolarly sequestered through the concerted actions of cystolic GSTs and the vacuolar GS-X pump in plants (Marrs *et al.*, 1995, *Nature*, 375:397 and data contained herein), medicarpin, and 15 presumably other isoflavonoid phytoalexins, is equally strong a candidate.

These data suggest that the GSTs which are induced following the hypersensitive response to avirulent fungal pathogens likely serve to facilitate the vacuolar storage of antimicrobial compounds in the healthy cells surrounding the lesion.

#### 20 Transport of glutathionated anthocyanins and auxins by the vacuolar GS-X pump of plant cells

The data which are now described demonstrate that the vacuolar GS-X pumps of corn (*Zea mays*) roots and etiolated hypocotyls of mung bean (*Vigna radiata*) transport the anthocyanin cyanidin-3-glucoside (C3G), and the phytohormone, indole- 25 3-acetic acid (IAA), after conjugation with glutathione. Whereas the unconjugated forms of these compounds undergo negligible uptake into vacuolar membrane vesicles, both C3G-GS and IAA-GS are subject to high rates of MgATP-dependent, uncoupler-insensitive uptake (Figure 27 and Table 12). IAA-GS and C3G-GS uptake approximates Michaelis-Menten kinetics to yield  $K_m$  values in the micromolar range

and  $V_{\max}$  values 7- to 40-fold greater than those measured with the artificial transport substrate, DNP-GS (Table 12 and Li *et al.*, 1995, *supra*). Uptake of both conjugates is inhibited by DNP-GS and vanadate in a manner consistent with mediation by the GS-X pump (Figure 27 and Table 13). In contrast, glutathionated abscissic acid (ABA-GS) is a poor substrate for the GS-X pump: uptake is relatively slow and only saturates at high substrate concentrations (Figure 27 and Table 13).

Table 12. Summary of kinetic parameters for MgATP-dependent, uncoupler-insensitive uptake of C3G-GS, IAA-GS and ABA-GS by vacuolar membrane vesicles purified from etiolated hypocotyls of *V. radiata* and roots of *Z. mays*. Kinetic parameters ( $K_m$ ,  $\mu\text{M}$ ;  $V_{\max}$ ,  $\text{nmol/mg/10 min}$ ) were computed from the data shown in Figure 27 by nonlinear least squares analysis. Values shown are means  $\pm$  SE.

PARAMETER	C3G-GS		IAA-GS
	<i>V. radiata</i>	<i>Z. mays</i>	<i>V. radiata</i>
$K_m$	$45.7 \pm 14.0$	$39.5 \pm 16.6$	$36.0 \pm 16.7$
$V_{\max}$	$45.3 \pm 6.5$	$79.1 \pm 14.7$	$17.7 \pm 5.8$
PARAMETER	IAA-GS	ABA-GS	
	<i>Z. mays</i>	<i>V. Radiata</i>	<i>Z. mays</i>
$K_m$	$47.7 \pm 19.6$	>1000	$128.8 \pm 79.1$
$V_{\max}$	$30.0 \pm 4.9$	$22.9 \pm 9.2$	$4.0 \pm 1.4$

It has been known for some time that the characteristic bronze coloration of *Bronze-2* (*bz2*) mutants is a consequence of the accumulation of cyanidin-3-glucoside in the cytosol. In wild type (*Bz2*) plants, anthocyanins are transported into the vacuole and become purple or red whereas in *bz2* plants, anthocyanin is restricted to the cytoplasm where it is oxidized to a brown ("bronze") pigment. However, until the present invention, the exact molecular basis of this lesion was unknown. Since *Bz2* encodes a GST responsible for conjugating anthocyanin with GSH (Marrs *et al.*, 1995) and glutathionated anthocyanins are transported by the vacuolar GS-X pump, the

experiments described herein explain the bronze phenotype. Being defective in the glutathionation of anthocyanins, *bz2* mutants are unable to pump these pigments from the cytosol into the vacuole lumen; a conclusion borne out by the ability of the GS-X pump inhibitor, vanadate, to phenocopy the *bz2* lesion in wild type protoplasts and the efficacy of cyanidin-3-glucoside-GS as a substrate for the plant vacuolar GS-X pump *in vitro*, as the data presented herein establish.

The concept underlying the above-described experiments on phytohormones is that they may be subject to metabolic interconversions and compartmentation analogous to those deduced for anthocyanins. On the one hand, it is now established that C3G must be glutathionated before it can be transported into the vacuole. On the other hand, it is evident that most of the vacuolar anthocyanins of intact plants are not stored in this form. Instead, they are subject to long term storage as their malonyl derivatives. It is therefore apparent that while C3G-GS is a short-lived but necessary intermediate for vacuolar anthocyanin compartmentation, it is not the terminal product of this process. The experiments with auxins further illustrate this principle by demonstrating that IAA is susceptible to glutathionation and that the resultant IAA-GS conjugate is transported by the vacuolar GS-X pump in a MgATP-dependent, uncoupler-insensitive, vanadate-inhibitible manner. Thus, even though IAA-GS derivatives have not been detected *in planta*, this does not exclude the possibility that they are short-lived transport intermediates necessary for subsequent vacuolar processing of this class of compounds.

<u>Table 13. Concentrations of DNP-GS and vanadate required to inhibit MgATP-dependent, uncoupler-insensitive uptake of C3G-GS, IAA-GS and ABA-GS by 50% (<math>I_{50}</math> values) by vacuolar membrane vesicles purified from etiolated hypocotyls of <i>V. radiata</i> and roots of <i>Z. mays</i>. <math>I_{50}</math> values (<math>\mu</math>M) were estimated by nonlinear least squares analysis after fitting the data to a single negative exponential. ND, not determined.</u>			
COMPOUND	C3G-GS	IAA-GS	ABA-GS
	<i>V. radiata</i>	<i>Z. mays</i>	<i>V. radiata</i>
Vanadate	7.9	8.2	6.5
DNP-GS	103.5	112.4	124.2
COMPOUND	C3G-GS	IAA-GS	ABA-GS
	<i>Z. mays</i>	<i>V. radiata</i>	<i>Z. mays</i>
Vanadate	5.5	ND	>150
DNP-GS	109.8	ND	231.5

#### Generation of a Transgenic Plant Comprising a Transgene Encoding a GS-X Pump

To generate a transgenic plant comprising a gene encoding YCF1, the following experiments were performed. The binary vector pROK-YCF1, encoding wild type YCF1 was constructed. The sense orientation of the inserts with respect to the CaMV 35S promoter of pROK (Baulscombe *et al.*, 1986, *Nature* 321:446-449) was confirmed and these constructs, as well as empty vector (pROK) controls, were transformed into *Agrobacterium* strain C58 by electroporation (Ausubel *et al.*, 1992, *Current Protocols in Molecular Biology*, pp 27-28).

Kanamycin-resistant *Agrobacterium* transformants were isolated, the integrity of the constructs in the bacterial recipient was established by PCR and *Arabidopsis* roots were inoculated with the transformants (Huang *et al.*, 1992, *Plant Mol. Biol.* 10:372-384). The resulting rosette shoots generated on selective medium



were transferred to root-inducing medium for regeneration. Stable insertion of the sense strands and constitutive expression of *YCF1* and *YCF1::HA* was demonstrated in the kanamycin-resistant *Arabidopsis* transformants, by probing Southern blots with *YCF1* and pROK sequences and by Northern analyses, respectively.

5           An association between *YCF1* expression and altered xenobiotic resistance was tested by screening multiple T2 generation pROK-*YCF1*, pROK-*YCF1::HA* and pROK empty vector transformant lines for tolerance to cadmium salts and the GS-conjugable xenobiotic 1-chloro-2,4-dinitrobenzene (CDNB). CDNB has three advantages for studies of this type: (i) It is an established plant toxin (Li *et al.*, 1995, *Plant Physiol.* 109:117-185); (ii) The kinetics of transport of its glutathionated  
10       derivative, DNP-GS, as well characterized for *YCF1* (Li *et al.*, 1996, *J. Biol. Chem.* 271:6509-6517) and the endogenous GS-X pump (Li *et al.*, 1995, *Plant. Physiol.* 107:1257-1268; Li *et al.*, 1995, *Plant Physiol.* 109:117-185). (iii) DNP-GS is the only known immediate metabolite of CDNB *in vivo* (Li *et al.*, 1995, *Plant Physiol.* 109:117-  
15       185).

          Methods similar to those described by Howden *et al.* (Howden *et al.*, 1992, *Plant Physiol.* 99:100-107; Howden *et al.*, 1995, *Plant Physiol.* 107:1059-1066; Howden *et al.*, 1995, *Plant Physiol.* 107:1067-1073) were applied to the initial  
20       characterization of the transformants. T2 seeds were first sown in rows on Cd<sup>2+</sup>-free and CDNB-free medium in Petri dishes standing on edge so that the roots grew vertically down the surface of the agar. Three to 4 days after germination, the seeds were transferred, again in rows, to media containing a range of CdSO<sub>4</sub> or CDNB concentrations. After rotating the Petri dishes though 180° and allowing growth for  
25       another 24-48 hours, the seedlings were scored for hook length. The results of this study are shown in Figure 28. It is evident from the data presented therein that transgenic *Arabidopsis* plants comprising *YCF1* acquire increased resistance to cadmium salts and the organic cytotoxin, CDNB.

          The disclosures of each and every patent, patent application and publication cited herein are hereby incorporated herein by reference in their entirety.

While this invention has been disclosed with reference to specific  
embodiments, it is apparent that other embodiments and variations of this invention  
may be devised by others skilled in the art without departing from the true spirit and  
scope of the invention. The appended claims are intended to be construed to include all  
5 such embodiments and equivalent variations.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

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Lu, Yu-Ping  
Li, Ze-Sheng

(ii) TITLE OF INVENTION: GLUTATHIONE-S-CONJUGATE TRANSPORT IN  
PLANTS

(iii) NUMBER OF SEQUENCES: 20

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

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## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5232 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TTATGAAAT TTATTATTT TGTGCTATG GTTTTTTGA ATTAGAAGCT CATTTCAAAG	60
TTGTTGATTT TCTTTCAGG GTAGGGAATT GGTGTGGTAG CTTGTGATGC ACTGTGTTTG	120
AGGGAAAGGA AAGGATAACG ATGGGGTTTG AGTTTATTGA ATGGTATTGT AAGCCGGTGC	180
CTAATGGTGT GTGGACTAAA ACAGTGCTA ATGCATTTGG TGCATACACG CCTGTGCTA	240
CTGACTCTTT TGTGCTTGGT ATCTCTCAAC TGGTCTGTGTT GGTTCTGTGC CTGTATCGTA	300
TATGGCTCGC CTAAAGGAT CACAAGGTGG AGAGGTTCTG TTTGAGGTCG AGATTGTATA	360
ACTATTTCTT GGCTTTGTTG GCTGGTATGC TACTGCTGAG CCTTTGTTTA GATTGATCAT	420
GGGGATTTC ATTTTAGATT TTGATGGACC TGGACTTCCT CCTTTGAGG CATTCGGATT	480
GGGTGTCAA GCTTTTGCTT GGGGCGCTGT AATGGTCATG ATTTTAATGG AACTAAAAAT	540
TTACATCCGT GAACTCCGTT GGTATGTCAG GTTTGCTGTC ATATATGCTC TTGTGGGGGA	600
TATGGTCTTG TTAAATCTTG TTCTCTCAGT CAAGGAGTAC TATAGCAGTT ATGTTCTGTA	660
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TCCCAATTTG GATACTTACC CTGGCTACAT GCCAGTCCGG AGTGAACTG TGGATGATTA	780
TGAGTATGAA GAGATTCTG ATGGACAACA AATATGCCCT GAGAAGCATC CAAATATATT	840
TGACAAAATC TTCTTCTCAT GGATGAATCC CTTGATGACT TTGGGATCTA AAAGGCCTCT	900
AACAGAGAAG GATGTGTGGT ATCTAGACAC TTGGGATCAG ACTGAACTC TGTTACGAG	960
TTTCCAGCAT TCCTGGGATA AGGAACTACA AAAGCCGCAA CCGTGGCTGT TGAGAGCATT	1020

GAACAATAGC CTGGGAGGAA GGTTTTGGTG GGGAGGATTT TGGAAGATCG GGAATGATTG 1080  
CTCACAGTTT GTGGGACCTC TTTTACTGAA TCAACTCTTA AAGTCAATGC AAGAGGATGC 1140  
GCCAGCTTGG ATGGGTTACA TCTATGCGTT CTCAATCTTT GGTGGAGTGG TGTTCCGGGT 1200  
GCTATGTGAA GCTCAATATT TCCAGAATGT CATGCGTGTT GGTTACCGAC TGAGATCTGC 1260  
TCTGATTGCT GCTGTGTTCC GCAAATCGTT GAGGTAACT AATGAAGGTC GTAGAAAGTT 1320  
TCAAACAGGA AAGATAACCA ACTTAATGAC GACTGATGCC GAATCTCTTC AGCAAATATG 1380  
CCAATCACTT CATAACATGT GGTCGGCTCC ATTTCTGATA ATTATAGCAC TGATTCTCCT 1440  
CTATCAGCAA TTGGGTGTTG CCTCGCTCAT TGGTGCAATG TTGTTGGTCC TTATGTTCCC 1500  
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## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 9936 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1621 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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Val-Trp Thr Lys Thr Val Ala Asn Ala Phe Gly Ala Tyr Thr Pro Cys
20           25           30
Ala Thr Asp Ser Phe Val Leu Gly Ile Ser Gln Leu Val Leu Leu Val
35           40           45
Leu Cys Leu Tyr Arg Ile Trp Leu Ala Leu Lys Asp His Lys Val Glu
50           55           60
Arg Phe Cys Leu Arg Ser Arg Leu Tyr Asn Tyr Phe Leu Ala Leu Leu
65           70           75           80
Ala Ala Tyr Ala Thr Ala Glu Pro Leu Phe Arg Leu Ile Met Gly Ile
85           90           95
Ser Val Leu Asp Phe Asp Gly Pro Gly Leu Pro Pro Phe Glu Ala Phe
100          105          110
Gly Leu Gly Val Lys Ala Phe Ala Trp Gly Ala Val Met Val Met Ile
115          120          125
Leu Met Glu Thr Lys Ile Tyr Ile Arg Glu Leu Arg Trp Tyr Val Arg
130          135          140
Phe Ala Val Ile Tyr Ala Leu Val Gly Asp Met Val Leu Leu Asn Leu
145          150          155          160
Val Leu Ser Val Lys Glu Tyr Tyr Ser Ser Tyr Val Leu Tyr Leu Tyr
165          170          175
Thr Ser Glu Val Gly Ala Gln Val Leu Phe Gly Ile Leu Leu Phe Met
180          185          190
His Leu Pro Asn Leu Asp Thr Tyr Pro Gly Tyr Met Pro Val Arg Ser
195          200          205
Glu Thr Val Asp Asp Tyr Glu Tyr Glu Glu Ile Ser Asp Gly Gln Gln
210          215          220
Ile Cys Pro Glu Lys His Pro Asn Ile Phe Asp Lys Ile Phe Phe Ser
225          230          235          240
Trp Met Asn Pro Leu Met Thr Leu Gly Ser Lys Arg Pro Leu Thr Glu
245          250          255

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Lys. Asp Val Trp Tyr Leu Asp Thr Trp Asp Gln Thr Glu Thr Leu Phe  
 260 265 270  
 Thr Ser Phe Gln His Ser Trp Asp Lys Glu Leu Gln Lys Pro Gln Pro  
 275 280 285  
 Trp Leu Leu Arg Ala Leu Asn Asn Ser Leu Gly Gly Arg Phe Trp Trp  
 290 295 300  
 Gly Gly Phe Trp Lys Ile Gly Asn Asp Cys Ser Gln Phe Val Gly Pro  
 305 310 315 320  
 Leu Leu Leu Asn Gln Leu Leu Lys Ser Met Gln Glu Asp Ala Pro Ala  
 325 330 335  
 Trp Met Gly Tyr Ile Tyr Ala Phe Ser Ile Phe Gly Gly Val Val Phe  
 340 345 350  
 Gly Val Leu Cys Glu Ala Gln Tyr Phe Gln Asn Val Met Arg Val Gly  
 355 360 365  
 Tyr Arg Leu Arg Ser Ala Leu Ile Ala Ala Val Phe Arg Lys Ser Leu  
 370 375 380  
 Arg Leu Thr Asn Glu Gly Arg Arg Lys Phe Gln Thr Gly Lys Ile Thr  
 385 390 395 400  
 Asn Leu Met Thr Thr Asp Ala Glu Ser Leu Gln Gln Ile Cys Gln Ser  
 405 410 415  
 Leu His Thr Met Trp Ser Ala Pro Phe Arg Ile Ile Ile Ala Leu Ile  
 420 425 430  
 Leu Leu Tyr Gln Gln Leu Gly Val Ala Ser Leu Ile Gly Ala Leu Leu  
 435 440 445  
 Leu Val Leu Met Phe Pro Leu Gln Thr Val Ile Ile Ser Lys Met Gln  
 450 455 460  
 Lys Leu Thr Lys Glu Gly Leu Gln Arg Thr Asp Lys Arg Ile Gly Leu  
 465 470 475 480  
 Met Asn Glu Val Leu Ala Ala Met Asp Thr Val Lys Cys Tyr Ala Trp  
 485 490 495  
 Glu Asn Ser Phe Gln Ser Lys Val Gln Thr Val Arg Asp Asp Glu Leu  
 500 505 510  
 Ser Trp Phe Arg Lys Ser Gln Leu Leu Gly Ala Leu Asn Met Phe Ile  
 515 520 525



Leu Asn Ser Ile Pro Val Leu Val Thr Ile Val Ser Phe Gly Val Phe  
 530 535 540  
 Thr Leu Leu Gly Gly Asp Leu Thr Pro Ala Arg Ala Phe Thr Ser Leu  
 545 550 555 560  
 Ser Leu Phe Ala Val Leu Arg Phe Pro Leu Phe Met Leu Pro Asn Ile  
 565 570 575  
 Ile Thr Gln Val Val Asn Ala Asn Val Ser Leu Lys Arg Leu Glu Glu  
 580 585 590  
 Val Leu Ala Thr Glu Glu Arg Ile Leu Leu Pro Asn Pro Pro Ile Glu  
 595 600 605  
 Pro Gly Glu Pro Ala Ile Ser Ile Arg Asn Gly Tyr Phe Ser Trp Asp  
 610 615 620  
 Ser Lys Gly Asp Arg Pro Thr Leu Ser Asn Ile Asn Leu Asp Val Pro  
 625 630 635 640  
 Leu Gly Ser Leu Val Ala Val Val Gly Ser Thr Gly Glu Gly Lys Thr  
 645 650 655  
 Ser Leu Ile Ser Ala Ile Leu Gly Glu Leu Pro Ala Thr Ser Asp Ala  
 660 665 670  
 Ile Val Thr Leu Arg Gly Ser Val Ala Tyr Val Pro Gln Val Ser Trp  
 675 680 685  
 Ile Phe Asn Ala Thr Val Arg Asp Asn Ile Leu Phe Gly Ser Pro Phe  
 690 695 700  
 Asp Arg Glu Lys Tyr Glu Arg Ala Ile Asp Val Thr Ser Leu Lys His  
 705 710 715 720  
 Asp Leu Glu Leu Leu Pro Gly Gly Asp Leu Thr Glu Ile Gly Glu Arg  
 725 730 735  
 Gly Val Asn Ile Ser Gly Gly Gln Lys Gln Arg Val Ser Met Ala Arg  
 740 745 750  
 Ala Val Tyr Ser Asn Ser Asp Val Tyr Ile Phe Asp Asp Pro Leu Ser  
 755 760 765  
 Ala Leu Asp Ala His Val Gly Gln Gln Val Phe Glu Lys Cys Ile Lys  
 770 775 780  
 Arg Glu Leu Gly Gln Lys Thr Arg Val Leu Val Thr Asn Gln Leu His  
 785 790 795 800

Phe Leu Ser Gln Val Asp Arg Ile Val Leu Val His Glu Gly Thr Val  
 805 810 815  
 Lys Glu Glu Gly Thr Tyr Glu Glu Leu Ser Ser Asn Gly Pro Leu Phe  
 820 825 830  
 Gln Arg Leu Met Glu Asn Ala Gly Lys Val Glu Glu Tyr Ser Glu Glu  
 835 840 845  
 Asn Gly Glu Ala Glu Ala Asp Gln Thr Ala Glu Gln Pro Val Ala Asn  
 850 855 860  
 Gly Asn Thr Asn Gly Leu Gln Met Asp Gly Ser Asp Asp Lys Lys Ser  
 865 870 875 880  
 Lys Glu Gly Asn Lys Lys Gly Gly Lys Ser Val Leu Ile Lys Gln Glu  
 885 890 895  
 Glu Arg Glu Thr Gly Val Val Ser Trp Arg Val Leu Lys Arg Tyr Gln  
 900 905 910  
 Asp Ala Leu Gly Gly Ala Trp Val Val Met Met Leu Leu Leu Cys Tyr  
 915 920 925  
 Val Leu Thr Glu Val Phe Arg Val Thr Ser Ser Thr Trp Leu Ser Glu  
 930 935 940  
 Trp Thr Asp Ala Gly Thr Pro Lys Ser His Gly Pro Leu Phe Tyr Asn  
 945 950 955 960  
 Leu Ile Tyr Ala Leu Leu Ser Phe Gly Gln Val Leu Val Thr Leu Thr  
 965 970 975  
 Asn Ser Tyr Trp Leu Ile Met Ser Ser Leu Tyr Ala Ala Lys Lys Leu  
 980 985 990  
 His Asp Asn Met Leu His Ser Ile Leu Arg Ala Pro Met Ser Phe Phe  
 995 1000 1005  
 His Thr Asn Pro Leu Gly Arg Ile Ile Asn Arg Phe Ala Lys Asp Leu  
 1010 1015 1020  
 Gly Asp Ile Asp Arg Thr Val Ala Val Phe Val Asn Met Phe Met Gly  
 1025 1030 1035 1040  
 Gln Val Ser Gln Leu Leu Ser Thr Val Val Leu Ile Gly Ile Val Ser  
 1045 1050 1055  
 Thr Leu Ser Leu Trp Ala Ile Met Pro Leu Leu Val Leu Phe Tyr Gly  
 1060 1065 1070

Ala Tyr Leu Tyr Tyr Gln Asn Thr Ala Arg Glu Val Lys Arg Met Asp  
 1075 1080 1085

Ser Ile Ser Arg Ser Pro Val Tyr Ala Gln Phe Gly Glu Ala Leu Asn  
 1090 1095 1100

Gly Leu Ser Thr Ile Arg Ala Tyr Lys Ala Tyr Asp Arg Met Ala Asp  
 1105 1110 1115 1120

Ile Asn Gly Arg Ser Met Asp Asn Asn Ile Arg Phe Thr Leu Val Asn  
 1125 1130 1135

Met Gly Ala Asn Arg Trp Leu Gly Ile Arg Leu Glu Thr Leu Gly Gly  
 1140 1145 1150

Leu Met Ile Trp Leu Thr Ala Ser Phe Ala Val Met Gln Asn Gly Arg  
 1155 1160 1165

Ala Glu Asn Gln Gln Ala Phe Ala Ser Thr Met Gly Leu Leu Leu Ser  
 1170 1175 1180

Tyr Ala Leu Asn Ile Thr Ser Leu Leu Thr Gly Val Leu Arg Leu Ala  
 1185 1190 1195 1200

Ser Leu Ala Glu Asn Ser Leu Asn Ala Val Glu Arg Val Gly Asn Tyr  
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Ile Glu Ile Pro Pro Glu Ala Pro Pro Val Ile Glu Asn Asn Arg Pro  
 1220 1225 1230

Pro Pro Gly Trp Pro Ser Ser Gly Ser Ile Lys Phe Glu Asp Val Val  
 1235 1240 1245

Leu Arg Tyr Arg Pro Gln Leu Pro Pro Val Leu His Gly Val Ser Phe  
 1250 1255 1260

Phe Ile His Pro Thr Asp Lys Val Gly Ile Val Gly Arg Thr Gly Ala  
 1265 1270 1275 1280

Gly Lys Ser Ser Leu Leu Asn Ala Leu Phe Arg Ile Val Glu Val Glu  
 1285 1290 1295

Glu Gly Arg Ile Leu Ile Asp Asp Cys Asp Val Gly Lys Phe Gly Leu  
 1300 1305 1310

Met Asp Leu Arg Lys Val Leu Gly Ile Ile Pro Gln Ser Pro Val Leu  
 1315 1320 1325

Phe Ser Gly Thr Val Arg Phe Asn Leu Asp Pro Phe Gly Glu His Asn  
 1330 1335 1340

Asp Ala Asp Leu Trp Glu Ser Leu Glu Arg Ala His Leu Lys Asp Thr  
 1345 1350 1355 1360  
 Ile Arg Arg Asn Pro Leu Gly Leu Asp Ala Glu Val Ser Glu Ala Gly  
 1365 1370 1375  
 Glu Asn Phe Ser Val Gly Gln Arg Gln Leu Leu Ser Leu Ser Arg Ala  
 1380 1385 1390  
 Leu Leu Arg Arg Ser Lys Ile Leu Val Leu Asp Glu Ala Thr Ala Ala  
 1395 1400 1405  
 Val Asp Val Arg Thr Asp Ala Leu Ile Gln Lys Thr Ile Arg Glu Glu  
 1410 1415 1420  
 Phe Lys Ser Cys Thr Met Leu Ile Ile Ala His Arg Leu Asn Thr Ile  
 1425 1430 1435 1440  
 Ile Asp Cys Asp Lys Ile Leu Val Leu Asp Ser Gly Arg Val Gln Glu  
 1445 1450 1455  
 Phe Ser Ser Pro Glu Asn Leu Leu Ser Asn Glu Gly Ser Ser Phe Ser  
 1460 1465 1470  
 Lys Met Val Gln Ser Thr Gly Ala Ala Asn Ala Glu Tyr Leu Arg Ser  
 1475 1480 1485  
 Leu Val Leu Asp Asn Lys Arg Ala Lys Asp Asp Ser His His Leu Gln  
 1490 1495 1500  
 Gly Gln Arg Lys Trp Ala Ser Ser Arg Trp Ala Ala Ala Ala Gln Phe  
 1505 1510 1515 1520  
 Ala Leu Ala Ala Ser Leu Thr Ser Ser His Asn Asp Leu Gln Ser Leu  
 1525 1530 1535  
 Glu Ile Glu Asp Asp Ser Ser Ile Leu Lys Arg Thr Asn Asp Ala Val  
 1540 1545 1550  
 Val Thr Leu Arg Ser Val Leu Glu Gly Lys His Asp Lys Glu Ala Glu  
 1555 1560 1565  
 Ser Leu Glu Glu His Asn Ile Ser Arg Glu Gly Trp Leu Ser Ser Leu  
 1570 1575 1580  
 Tyr Arg Met Val Glu Gly Leu Ala Val Met Ser Arg Leu Ala Arg Asn  
 1585 1590 1595 1600  
 Arg Met Gln Gln Pro Asp Tyr Asn Phe Glu Gly Asn Thr Phe Asp Trp  
 1605 1610 1615

Asp Asn Val Glu Met  
1620

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5175 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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GAATTCGCGG CCGCCGGCGA ATTTGCACTC TTTACCTCTC TTGACTCCG TGAGATTCGA      60
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ACTAAACTG TGGATTATGC GTTTGGTGCA TACACGCCTT GTGCTATTGA CTCTTTTGTG      240
CTTGGTATCT CTCATCTGGT TCTGTTGATT CTGTGTCTTT ATCGCTTGTG GCTCATCACC      300
AAGGATCACA AAGTGGATAA GTTCTGCTTG AGGTCTAAAT GGTTCAGCTA TTTCTGGCT      360
CTTTTGGCTG CTTATGCTAC TGCGGAGCCT TTGTTTAGAT TGGTCATGAG GATCTCTGTT      420
TTGGATTGAG ATGGAGCTGG GTTTCCTCCC TATGAGGCGT TTATGTTGGT CCTTGAGGCT      480
TTTGCTTGGG GTTCTGCTTT GGTTCATGACT GTTGTGGAAA CTAAACGTA TATCCATGAA      540
CTCCGTTGGT ATGTCAGATT CGCTGTCATT TATGCTCTTG TGGGAGACAT GGTGTTGTTA      600
AATCTTGTTT TCTCTGTTAA GGAGTACTAT GGCAGTTTTA AACTGTATCT TTACATAAGC      660
GAGGTGGCAG TTCAGGTTGC ATTTGGAACC CTCTTGTTTG TGTATTTCCC TAATTGAGGAC      720
CCTTACCCTG GTTACACACC AGTTGGGACT GAAAATTCAG AGGATTACGA GTATGAAGAG      780
CTTCCTGGAG GAGAAAATAT ATGTCCTGAG AGGCATGCAA ATTTATTTGA CAGTATCTTC      840
TTCTCATGGT TGAACCCATT GATGACTCTG GGATCAAAAC GACCTCTCAC CGAGAAGGAT      900
GTATGGCATC TGGACACTTG GGATAAACT GAAACTCTTA TGAGGAGCTT CCAGAAGTCC      960

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TGGGATAAGG AACTAGAAAA GCCCAAACCG TGGCTTTTGA GAGCACTGAA CAACAGCCTT	1020
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GGTTACATCT ATGCAATCTC AATCTTTGTT GGAGTGGTAT TGGGGGTTTT ATGTGAAGCT	1200
CAGTATTTCC AAAATGTGAT GCGTGTGGT TACCGGCTTA GGTCTGCACT GATTGCTGCT	1260
GTGTTCCGAA AATCTTTGAG GCTAACTAAT GAGGGGCGGA AGAAGTTTCA AACAGGAAAA	1320
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ACGTGTGCAT CTTAGATGAA CCATTGAGTG CCCTTGATGC GCATGTTGGT CAGCAGGTTT	2460

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TCGTTATATG CTACGTCTTG ACTCAAGTAT TTCGGGTTTC AAGCATCACT TGGTTGAGTG	2940
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CGCTTCTTTC GTTTGGACAG GTCTCTGTGA CATTGATCAA TTCATATTGG TTGATTATGT	3060
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TCTTGATTTT TCCAATGGAT AAGGTGGGAA TTGTTGGGAG GACAGGCGCT GGGAAATCAA	3960

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AAGCAACTGC TGCAGTTGAC GTAAGAACTG ATGTTCTCAT CCAAAGACC ATCCGAGAAG	4380
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CACAACCTTT AGAAGGTCAA AGGAAATGGC AAGCTTCTTC TCGTTGGGCT GCAGCTGCTC	4680
AATTTCATT GGCTGTGAGC CTCACTTCAT CTCACAACGA CCTCCAAAGC CTTGAAATCG	4740
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CGAGGAACAG AATGCAACAC CCGGATTACA ATTTAGAAGG GAAATCGTTT GACTGGGACA	4980
ATGTCGAGAT GTAAACGATG AAAGGCTTAC ACTAATAGAC CTAAAACTCC CATTTTGATG	5040
GAACTTTTAT TTGTATTGCT TGGGATACAC GTAACAAAT GCCCATTAAT CGTGGTGTA	5100
CTATATAGGC TATGCTTCTT TTGGGAAAAA GAGAGTTTGA TTACAGAGGA TGTGATGATA	5160
ACACAATTGG AATTC	5175

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10342 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear



(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGGAGGTTTG GTTTTTTCCC TATCAATCGA ATTCCAATTC GTGCTCGTAA CGTGGATTTT	60
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TCTCCATGAG AATGTATGCT TTTAACTTT TTTTTTTTG TTTTTCCT TCGGAGCTAA	180
CTTTGGGGGC TGGTCTCGGT CTCTGTTTC TCTCCACTAA AAAGATAAAA AGCTTTTGCC	240
ATCTTTTTTT TTTTCTCAAT AATCTATCAC ATCGTTTTT TTCTTTGTT TTTTCTCCAT	300
TTGTCTTCAT TGAGTTCATA GCCACATAAT TATTGATTC TTTTCTTTT AGTGTTCCTG	360
TTACTGATGC GTTTCATTAT TTATACTCT CACTTGCAGA TTCGAGGATT GTTAGTTTCT	420
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GAGCCGTTGG ATTGGTATTG CAAGCCGGTG CCGAATGGTG TGTGGACTAA AACTGTGGAT	540
TATGCGTTTG GTGCATACAC GCCTTGTGCT ATTGACTCTT TTGTGCTTGG TATCTCTCAT	600
CTGGTTCTGT TGATTCTGTG TCTTTATCGC TTGTGGCTCA TCACGAAGGA TCACAAAGTG	660
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GCTACTGCGG AGCCTTTGTT TAGATTGGTC ATGAGGATCT CTGTTTGGGA TTTGGATGGA	780
GCTGGGTTTC CTCCCTATGA GGTGTGTTAT CACTTGTGCTG TTTGTGAT GTTGTCTCC	840
TTCTGTATGT TTTTCTCTGA GAGATGCTGT TGTTTTGTGC TTTATTGGC AGGCGTTTAT	900
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GCGTCATTTT TTTTTGTTT TTGGCAGTTT TAAACTGTAT CTTTACATAA GCGAGGTGGC	1200
AGTTCAGGTT TGCACTTTAA AACTCCTTTT TGCACTCTCC AACTACTCT TTACCATGTG	1260
CTGTATCTAA GTCACACTGT AAATGATACA ACTTTGTTTT TATAATGACG TTAAGGATGG	1320

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TTACCCTGGT TACACACCAG TTGGGACTGA AAATCCGAG GATTACGAGT ATGAAGAGCT	1440
TCCTGGAGGA GAAAATATAT GTCCTGAGAG GCATGCAAAT TTATTTGACA GTATGTCACT	1500
CTACACTTCT CATTCCCTAC TTTGTTTTTA TAGGTGCATT TTCTATTTTA ATTGTGAGAA	1560
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GGTCATTAAA ATTGTTTCCG CAGAGAGTAG TCTATAGTCT CTTCAAAATT TTTTTTTGAC	1740
TTATCCTCCC GTTCTCCCTA GAAATGAACT TATGATTTGT GACTGTGCCG AGGTTTTTGC	1800
TTAGTGATCA TCACTTCGAC TAAGCTGCAA CATTTTATAT AGTATATTCG TCAACATTG	1860
TCAAACCTTG ACTATTATGT TCCTTCTTAC CCTTGTCTTT CAACCCACAG GTATCTTCTT	1920
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TAACAACGTG TCTCATACTG TCTATGACTG GCATGGTTGC GTGACATATT TTTATCTCAT	2100
TTTTTAGCTT CCAGAAGTCC TGGGATAAGG AACTAGAAAA GCCCAAACCG TGGCTTTTGA	2160
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ATTCTTTTCT TTCTTGCTAG GACTTCATGG TATTTTGGT CTAGAGTAGA TGCTACGAAT	3120
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GAAAATAGCC TGATTCTACT CTTAGAGTTA GTTTTTTTG TTTGTTAATA CATTTCATG	3240
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CCTGTACTGT TATGTCTGTT GAAAGCTATA GCAGATGCTT ATAGATTGCT TCATTCTGCT	3540
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10342

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1622 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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Met Gly Phe Glu Pro Leu Asp Trp Tyr Cys Lys Pro Val Pro Asn Gly
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Ala Ile Asp Ser Phe Val Leu Gly Ile Ser His Leu Val Leu Leu Ile
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Leu Cys Leu Tyr Arg Leu Trp Leu Ile Thr Lys Asp His Lys Val Asp
        50              55              60

Lys Phe Cys Leu Arg Ser Lys Trp Phe Ser Tyr Phe Leu Ala Leu Leu
65              70              75              80

Ala Ala Tyr Ala Thr Ala Glu Pro Leu Phe Arg Leu Val Met Arg Ile
                85              90              95

Ser Val Leu Asp Leu Asp Gly Ala Gly Phe Pro Pro Tyr Glu Ala Phe
                100              105              110

Met Leu Val Leu Glu Ala Phe Ala Trp Gly Ser Ala Leu Val Met Thr
        115              120              125

Val Val Glu Thr Lys Thr Tyr Ile His Glu Leu Arg Trp Tyr Val Arg
        130              135              140

Phe Ala Val Ile Tyr Ala Leu Val Gly Asp Met Val Leu Leu Asn Leu
145              150              155              160

Val Leu Ser Val Lys Glu Tyr Tyr Gly Ser Phe Lys Leu Tyr Leu Tyr
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Ile Ser Glu Val Ala Val Gln Val Ala Phe Gly Thr Leu Leu Phe Val  
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 Tyr Phe Pro Asn Leu Asp Pro Tyr Pro Gly Tyr Thr Pro Val Gly Thr  
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 Ile Cys Pro Glu Arg His Ala Asn Leu Phe Asp Ser Ile Phe Phe Ser  
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 Lys Asp Val Trp His Leu Asp Thr Trp Asp Lys Thr Glu Thr Leu Met  
 260 265 270  
 Arg Ser Phe Gln Lys Ser Trp Asp Lys Glu Leu Glu Lys Pro Lys Pro  
 275 280 285  
 Trp Leu Leu Arg Ala Leu Asn Asn Ser Leu Gly Gly Arg Phe Trp Trp  
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 Gly Gly Phe Trp Lys Ile Gly Asn Asp Cys Ser Gln Phe Val Gly Pro  
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 Glu Asn Ser Phe Gln Ser Lys Val Gln Thr Val Arg Asp Asp Glu Leu  
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 Ser Trp Phe Arg Lys Ala Gln Leu Leu Ser Ala Phe Asn Met Phe Ile  
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 Phe Leu Ser Gln Val Asp Lys Ile Leu Leu Val His Glu Gly Thr Val  
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 820 825 830  
 Pro Arg Leu Met Glu Asn Ala Gly Lys Val Glu Asp Tyr Ser Glu Glu  
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 Asn Gly Glu Ala Glu Val His Gln Thr Ser Val Lys Pro Val Glu Asn  
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 Gly Asn Ala Asn Asn Leu Gln Lys Asp Gly Ile Glu Thr Lys Asn Ser  
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 Phe Arg Val Ser Ser Ile Thr Trp Leu Ser Glu Trp Thr Asp Ser Gly  
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 Thr Pro Lys Thr His Gly Pro Leu Phe Tyr Asn Ile Val Tyr Ala Leu  
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 Leu Ser Phe Gly Gln Val Ser Val Thr Leu Ile Asn Ser Tyr Trp Leu  
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 Ile Met Ser Ser Leu Tyr Ala Ala Lys Lys Met His Asp Ala Met Leu  
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Gly Ser Ile Leu Arg Ala Pro Met Val Phe Phe Gln Thr Asn Pro Leu  
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 Gly Arg Ile Ile Asn Arg Phe Ala Lys Asp Met Gly Asp Ile Asp Arg  
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 Pro Val Tyr Ala Gln Phe Gly Glu Ala Leu Asn Gly Leu Ser Ser Ile  
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 Arg Phe Asn Leu Asp Pro Phe Ser Glu His Asn Asp Ala Asp Leu Trp  
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 1490 1495 1500  
 Gln Arg Lys Trp Gln Ala Ser Ser Arg Trp Ala Ala Ala Ala Gln Phe  
 1505 1510 1515 1520  
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 1525 1530 1535

Glu Ile Glu Asp Asp Asn Ser Ile Leu Lys Lys Thr Lys Asp Ala Val  
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 Val Thr Leu Arg Ser Val Leu Glu Gly Lys His Asp Lys Glu Ile Glu  
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 1585 1590 1595 1600  
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 Trp Asp Asn Val Glu Met  
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## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1251 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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 CTAATATCCT TTTCTCAAAA GAAAAAATG TTTGTAACT TGGACACGAT ATTATTATCC 780  
 AACTTCCTTT CTAGATATTC ATTTTAAAT TACCTATATA TTTTATTTT CTCAAAATAT 840  
 ACTAAAAATT GGATAGAGCT ATTAAATAAA AAAGATAGAA TTTAGAGAGA AATAGCAACA 900  
 TAATGAATTA TAATATAAAT ATTTTGTAAG GAAATAACAA ACTTTATAGT TAGTTTGCCT 960  
 AATATAGAAA AAAGATACAG TTATTTACCC ATTTGTTTGT GTGTAAAAA AGGAGTAAAA 1020  
 TAAACAGAGA AAAGAGCTTC TTGTTTTTAC TTGTGAACGT TATTGACTTT TCGGCCTCTC 1080  
 TCTCTTCTCT ATACAAATAT ATGGATCTTC ATTTCTTCGT ATAGTGTAAG CAGTGACGCA 1140  
 TCCATTTATC ATCATCTCCT TATAAATCTC GAATCTGCCA CAGAGAGAGC GTGTGACAAA 1200  
 ATGAGTTCAT AAGATTCCGT TATCGTCTTC CTGATTCCTC CAAATCTCCG G 1251

## (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1368 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AAACAATTGG TGTATTTTGA ATTTTTCATG CAACGCACGT GAACAGCTTA ATTGCTTGAT 60  
 TGGAAACAAA CCTTTTGA TAGATTAAAT CAGTTTTAGG TGTTTTGGAA AATTAACGAA 120  
 CTATAGTGGA GATTAATTAA TTTTATATTA GTCTTTTTTA GTACACAAAT CGAAGTTTCC 180  
 TAGATTTTTT CAAAGTTGAA AATAATATTG ATAATATTTA TCAACAATGA ATCTACAAAA 240  
 ACATAATTTT TTTGCCAAAC AAATAACACC GAAACAAGAT TCATTCACCTA TTTTGGTTT 300  
 AAAAAAAAAA ATCAAAATTA CACTATTATG AAGCCAATTT TTGTATGCAA AAAACCTGTA 360

TGTATCAATT TGTTTGTATT AAAAAGTAAG CATTTATGTC TTTTTTTTAT AAATAATAGA	420
AACACTTACT AGATGAATAG ATTTTTTGGT TTTAGAACAG AATACTATAA TTGTATTTAT	480
ATAGCTTTTT TATATTATTC GATATAGAAA AGTGTATATA TAGGAAAAAT GTACCATATA	540
CTGTCAATAA CATATTTGAT TCTAAATATA AATAGAATTG TTTTAAAGAA ATATGATCGT	600
TTATAATTAA ATGGTTTTTA ATGTCTTTTC TTGGGGCAAA AAACAAAGCT TGTCTTTTCGT	660
CCATATATTT GCATCGTAAG GGGTGACGTA TCACTCTCTC TTTCTCTCAA ATATTATTCT	720
TCAATCTCTT TTTGGGGAAT CTTGAGCAA ATTAGTGAGA GAACCCACCC ACTTTCTTTC	780
TCATATGAGT ACATAAGATC CCTTTTGAGT TTTCGTGTTT TGCCAAAATC TCCAGGTAAA	840
GCTTCTCCCT TTTTCTCTGT TTTCTCTGTT TTGTTATTCT CCCTTTTCTC CATTGTAGCT	900
TTTTCTGTA AAGTGGGATT GATAGTTTGG TTTCATGGAT TTCAAATTTG TGTTATTTGA	960
CTCGATACCA TCTTAAATGC AGAGTCTTTT CGTGATAATA AAATTATGGA TTCGTTTCAA	1020
AGTTTTTTTT TTTTCGTATG GAAAACACTT GAGCTCTCTC AATCTTGTAG TCTTGACTCT	1080
TGATGATTCT TCTATGTTCT CGTTGTGATT GCTTGTCACT GTTCTATCTT TATATATGAT	1140
TAAATGCAAT TTGCCCCCTT TTTACGCGCG AATGTATTTA TTATCTTTCG CACTCTGGGT	1200
CCATTTCTTG TCACTTGAGC ACATAATGAT TGATTTATGA CTTTTTAAAG TTATGAAAAT	1260
TTATTATTTT TGTTGCTATG GTTTTTTGGG ATTAGAAGCT CATTTCAAAG TTGTTGATTT	1320
TCTTTCAGG GTAGGGAATT GGTGTGGTAG CTTGTGATGC ACTGTGTT	1368

## (2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 6 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ala Cys Asp Glu Phe Gly



1

5

## (2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 6 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ala Cys Asp His Ile Lys  
1 5

## (2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 12 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTGACAATAT GC

12

## (2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 54 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GTTTCACAGT TTAAGCGTA GTCTGGGACG TCGTATGGGT AATTTTCATT GACC

54

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AAACTGCAGA TGGCTGGTAA TCTTGTTTC

29

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GCCTCTAGAT CAAGCGTAGT CTGGGACGTC GTATGGGTAA TTTTCATTGA

50

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AGATTAAAGCC ATGCATGTCT

20

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TGCTGGTACC AGACTTGCCC TCC

23

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GAAAGTGGAT GTGGGACGGG C

21

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TCCATATGTT TACTGGC

17

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AAACCGGTGC GGCCGCCATG GGGTTTGAGC CGT

33

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AATTAACCCT CACTAAAGGG

20

What is claimed is:

1. An isolated DNA encoding a plant GS-X pump polypeptide.
2. The isolated DNA of claim 1, wherein said DNA is selected from the group consisting of DNA comprising *AtMRP1* and *AtMRP2*, and any mutants, derivatives, homologs and fragments thereof encoding GS-X pump activity.
3. An isolated preparation of a polypeptide comprising a plant GS-X pump.
4. The isolated preparation of a polypeptide of claim 3, wherein said polypeptide is selected from the group consisting of *AtMRP1*, *AtMRP2*, and any mutants, derivatives, homologs and fragments thereof having GS-X pump activity.
5. A recombinant cell comprising the isolated DNA of claim 1.
6. The recombinant cell of claim 5, wherein said cell is selected from the group consisting of a prokaryotic cell and a eukaryotic cell.
7. A vector comprising the isolated DNA of claim 1.
8. An antibody specific for a plant GS-X pump polypeptide.
9. An isolated preparation of a nucleic acid which is in an antisense orientation to all or a portion of a plant GS-X pump gene.
10. A cell comprising the isolated preparation of a nucleic acid of claim 9.

11. A vector comprising the isolated preparation of a nucleic acid of claim 9.
12. A transgenic plant, the cells, seeds and progeny of which comprise an isolated DNA encoding a plant GS-X pump.
13. A transgenic plant, the cells, seeds and progeny of which comprise an isolated preparation of a nucleic acid which is in an antisense orientation to all or a portion of a plant GS-X pump gene.
14. A transgenic plant, the cells, seeds and progeny of which comprise an isolated DNA encoding YCF1, or any mutants, derivatives, homologs and fragments thereof having YCF1 activity.
15. An isolated DNA comprising a plant GS-X pump promoter sequence.
16. The isolated DNA of claim 15, wherein said promoter sequence is selected from the group consisting of an *AtMRP1* and an *AtMRP2* promoter sequence.
17. A cell comprising the isolated DNA of claim 15.
18. A vector comprising the isolated DNA of claim 15.
19. The isolated DNA of claim 15, further comprising a reporter gene operably fused thereto.
20. A transgenic plant, the cells, seeds and progeny of which comprise a transgene comprising an isolated DNA comprising GS-X pump promoter sequence.

21. A method of identifying a compound capable of affecting the expression of a plant GS-X gene comprising  
providing a cell comprising an isolated DNA comprising a plant GS-X pump promoter sequence having a reporter sequence operably linked thereto,  
adding to said cell a test compound, and  
measuring the level of reporter gene activity in said cell, wherein a higher or a lower level of reporter gene activity in said cell compared with the level of reporter gene activity in a cell to which the test compound was not added, is an indication that said test compound is capable of affecting the expression of a plant GS-X pump gene.

22. A method of removing xenobiotic toxins from soil comprising growing in the soil a transgenic plant of comprising an isolated DNA encoding a GS-X pump.

23. A method of removing heavy metals from soil comprising growing in the soil a transgenic plant of comprising an isolated DNA encoding a GS-X pump.

24. A method of generating a transgenic pathogen resistant plant comprising introducing to the cells of said plant an isolated DNA encoding a GS-X pump, wherein said pump is capable of transporting glutathionated isoflavonoid alexins into the cells of said plant.

25. A method of manipulating plant pigmentation comprising modulating the expression of a GS-X pump protein in said plant, wherein said GS-X pump protein is selected from the group consisting of AtMRP1, AtMRP2 and YCF1.

26. A method of alleviating oxidative stress in a plant comprising introducing into the cells of said plant DNA encoding a GS-X pump, wherein said DNA is selected from the group consisting of DNA encoding AtMRP1, AtMRP2 and YCF1.

27. A method of manipulating the expression of a gene in a plant cell comprising  
operably fusing a GS-X pump promoter sequence to the DNA sequence encoding said gene to form a chimeric DNA, and  
generating a transgenic plant, the cells of which comprise said chimeric DNA, wherein upon activation of said GS-X pump promoter sequence, the expression of said gene is manipulated.



1/41

Fig. 1A

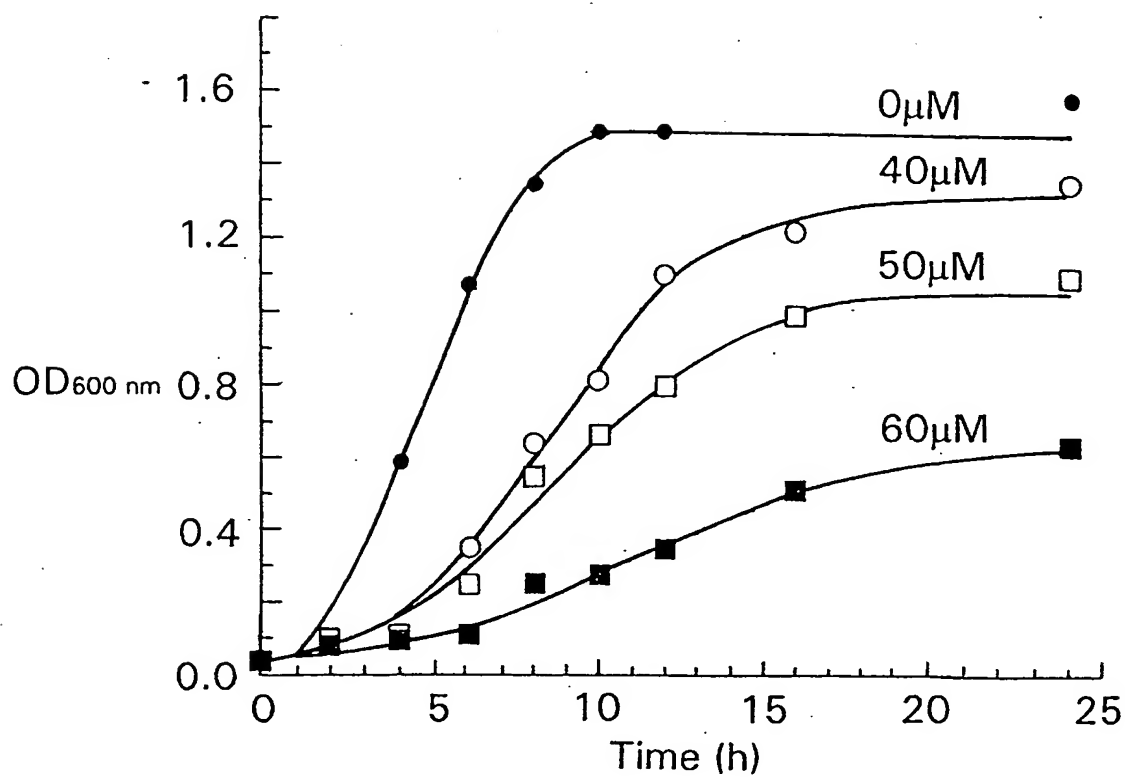
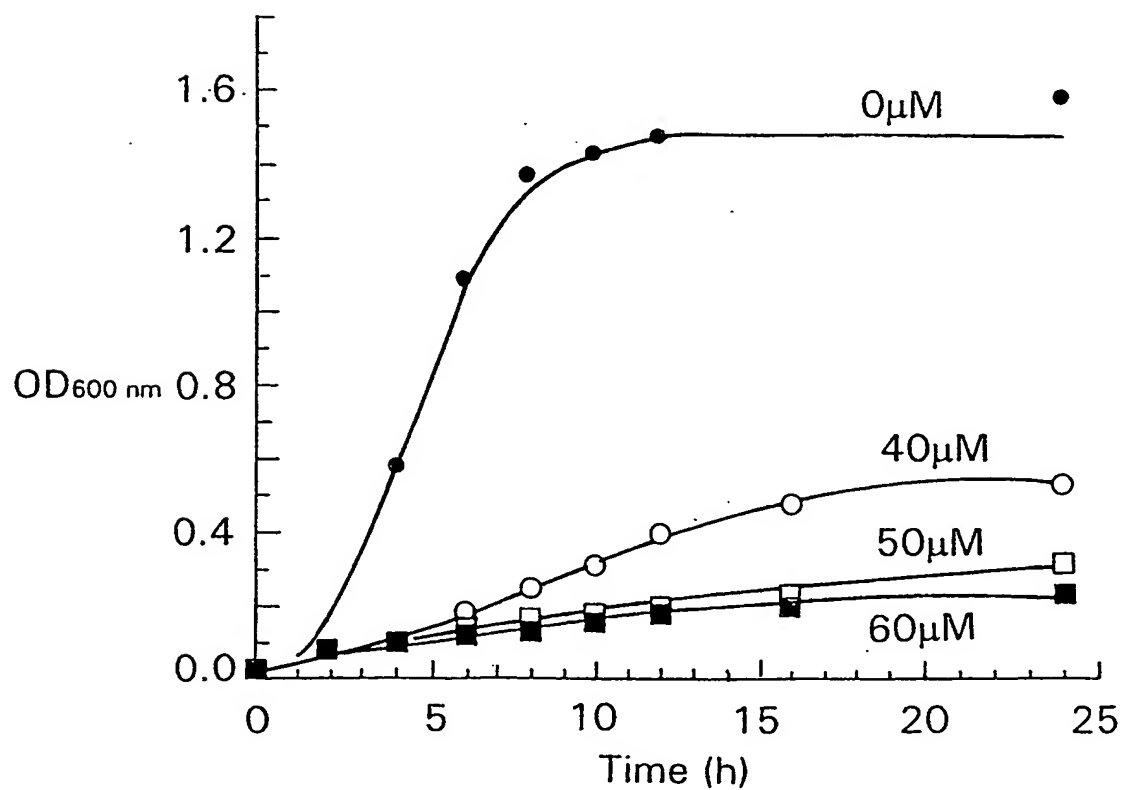
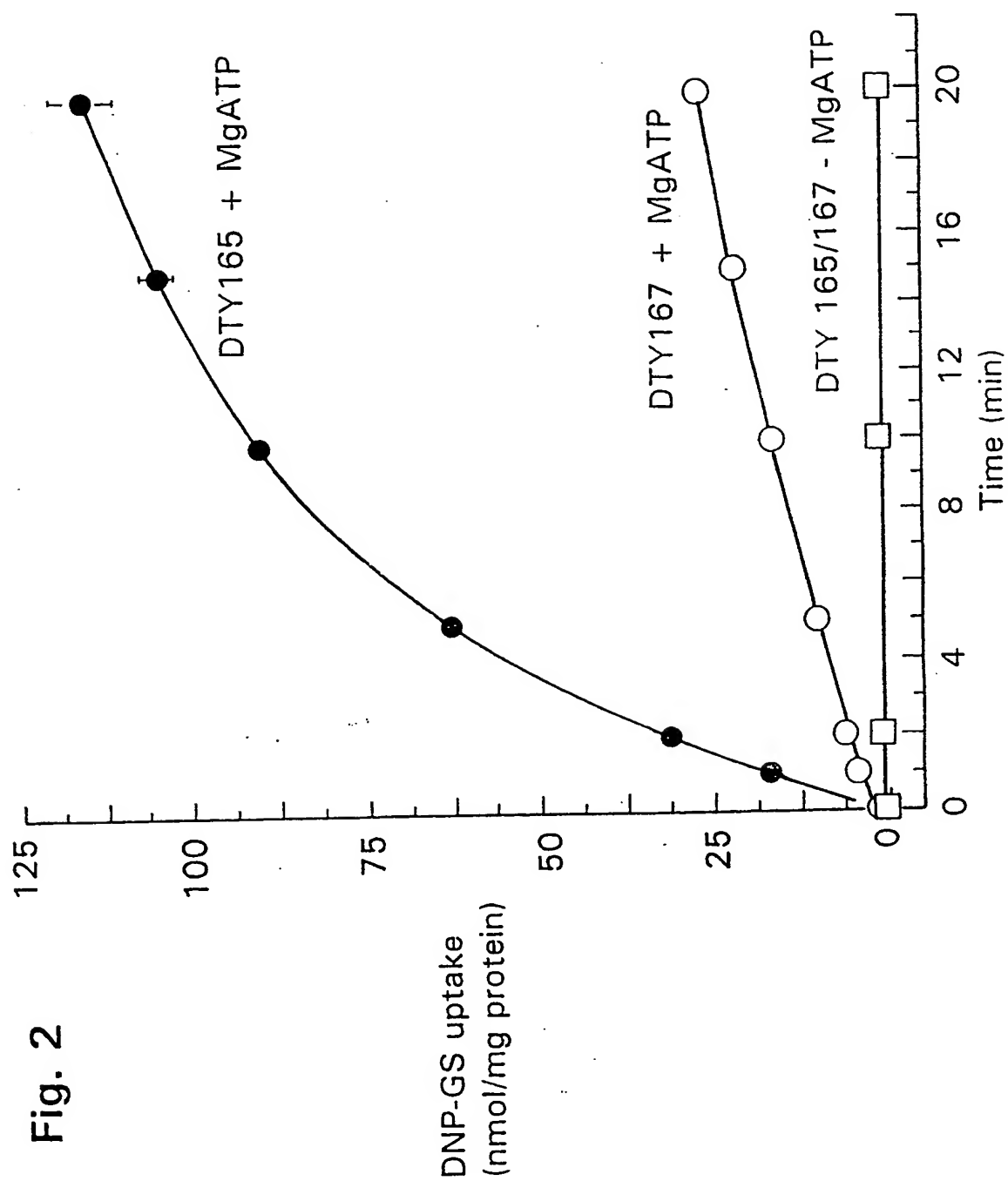


Fig. 1B



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2/41



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Fig. 3A

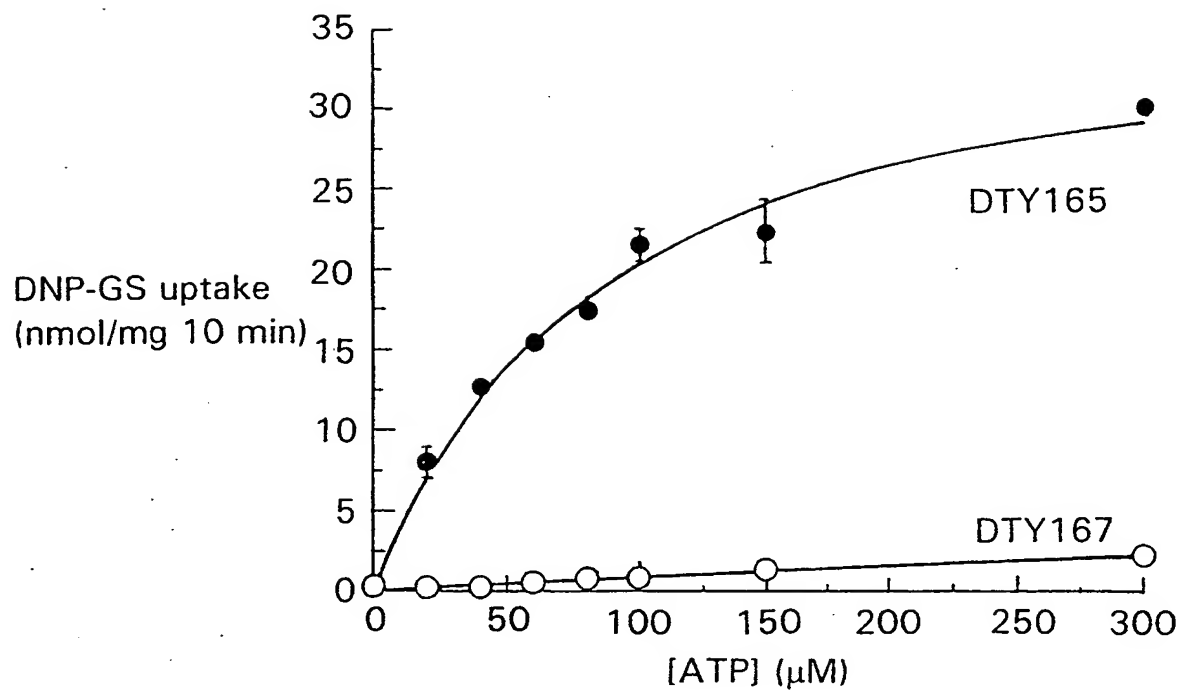
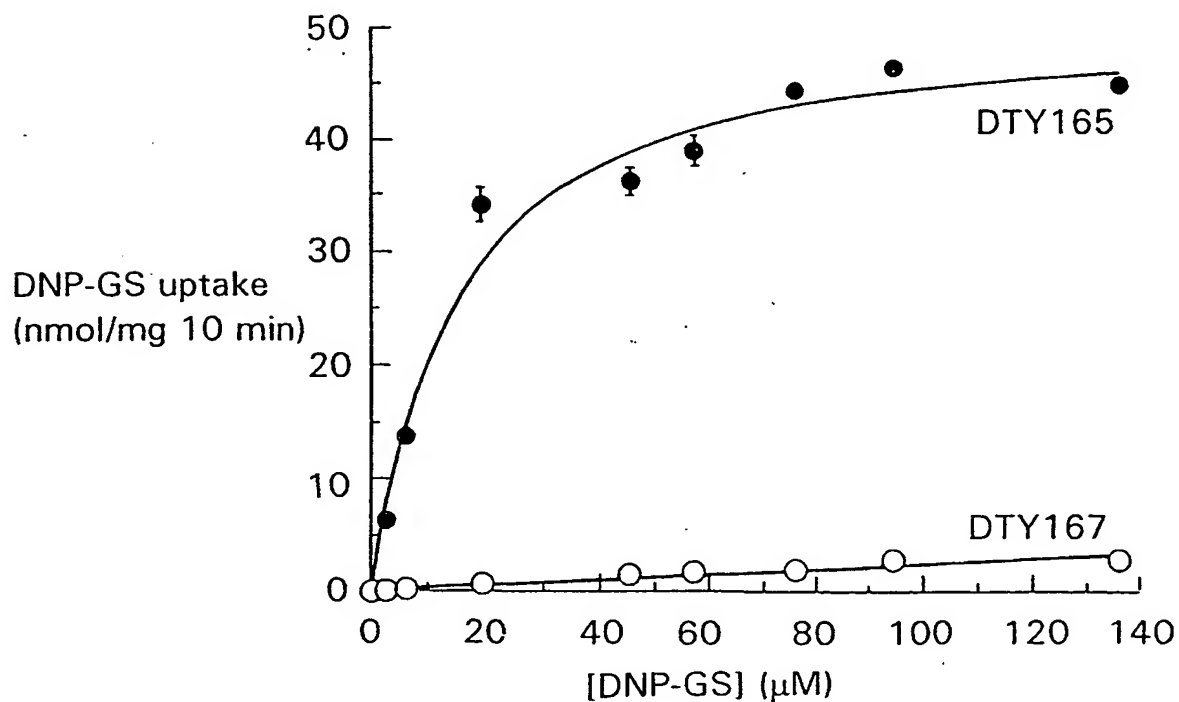


Fig. 3B



4/41

Fig. 4A

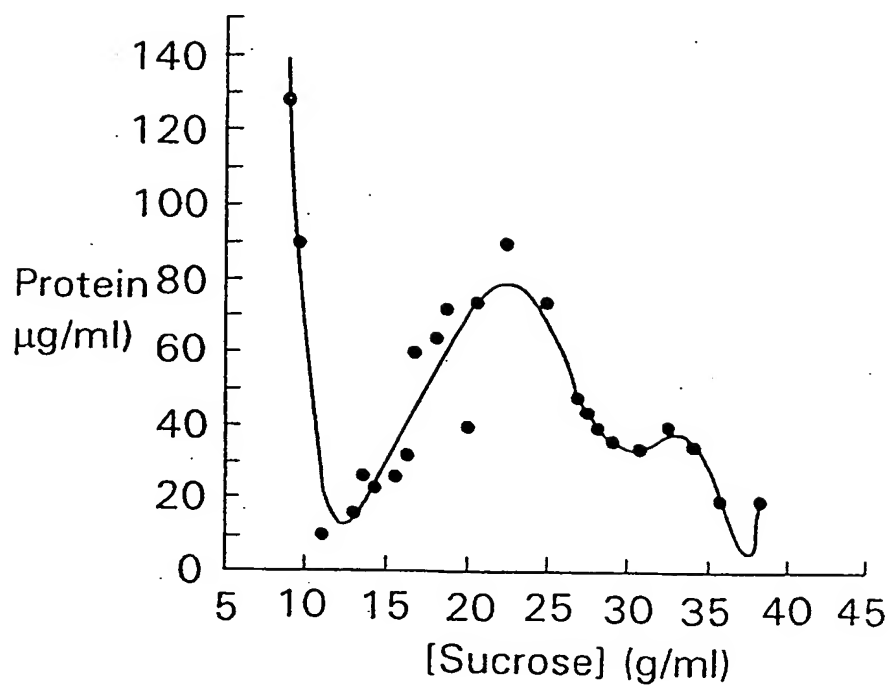
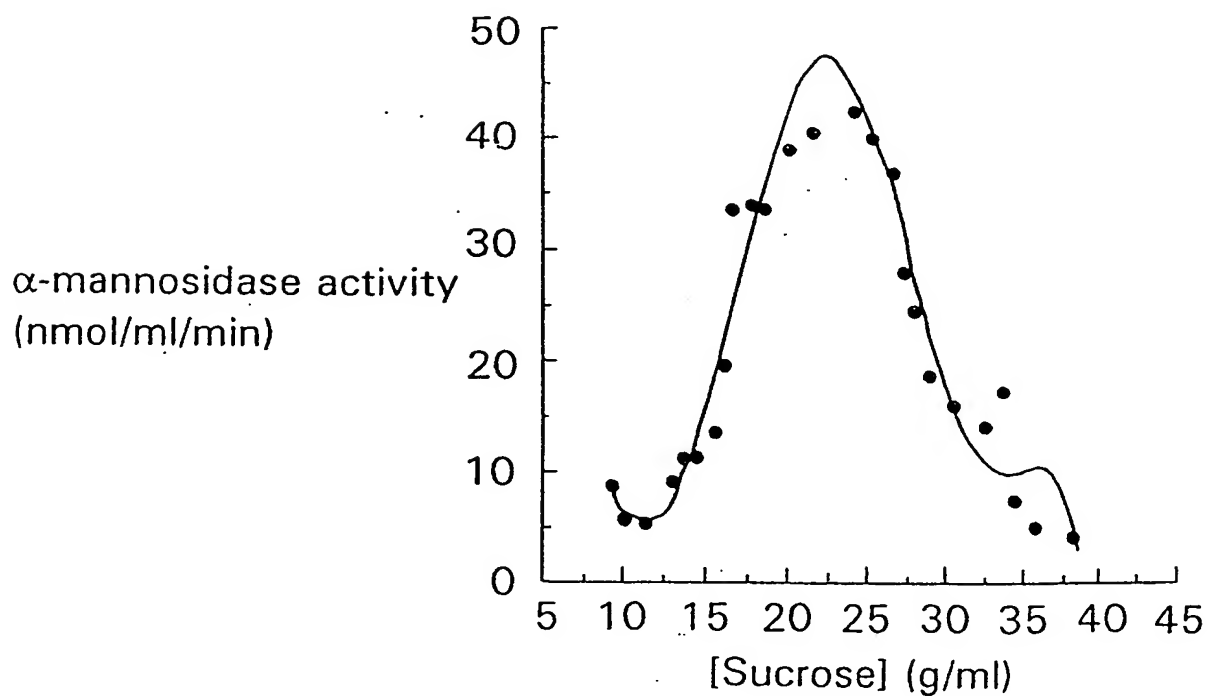
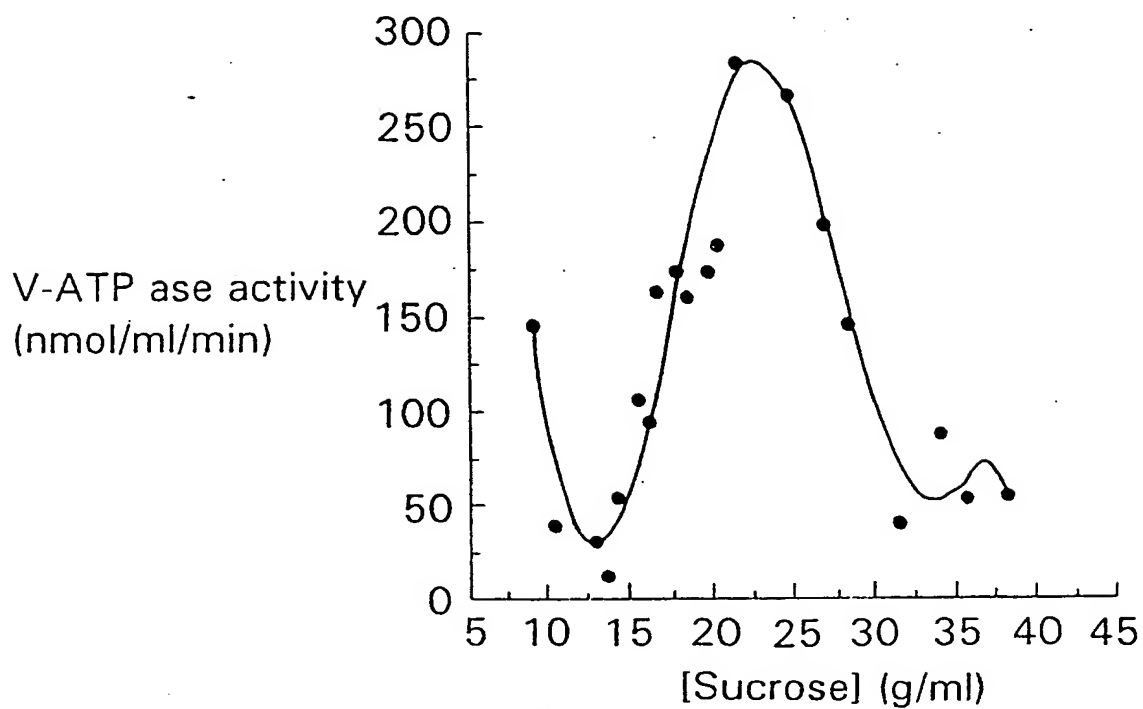
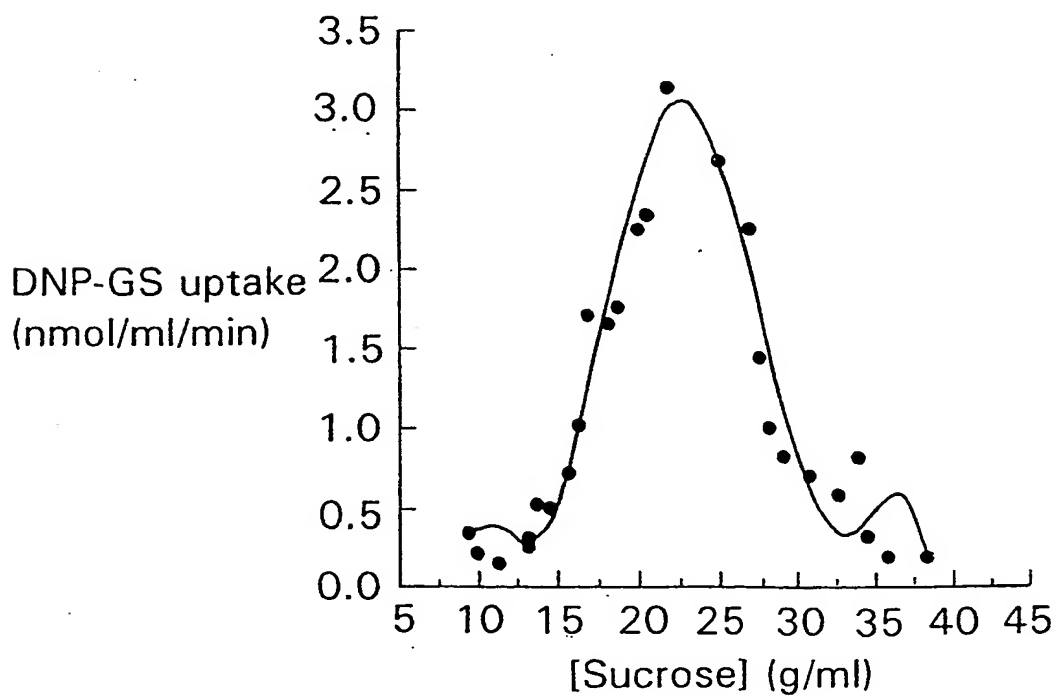


Fig. 4B



5/41

**Fig. 4C****Fig. 4D**

6/41

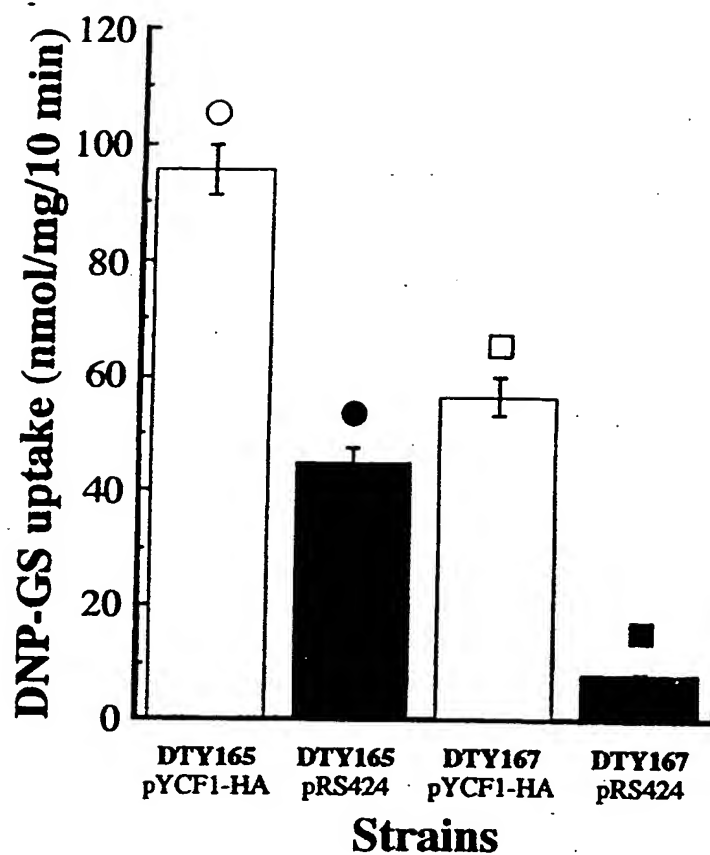


Fig. 5A

7/41

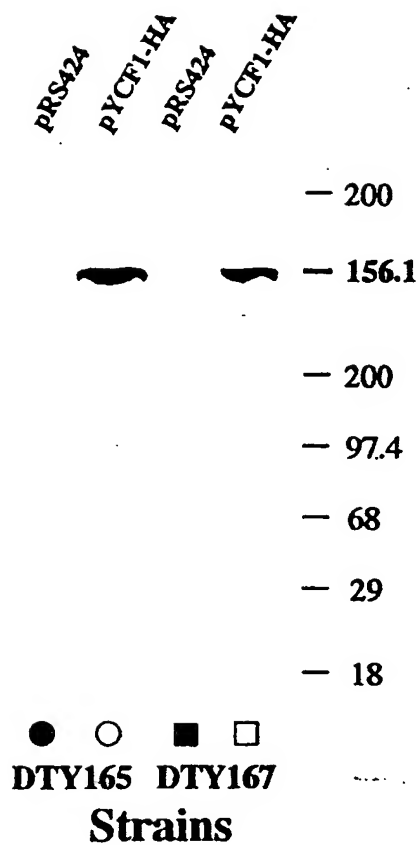


Fig. 5B

8/41

Fig. 6A

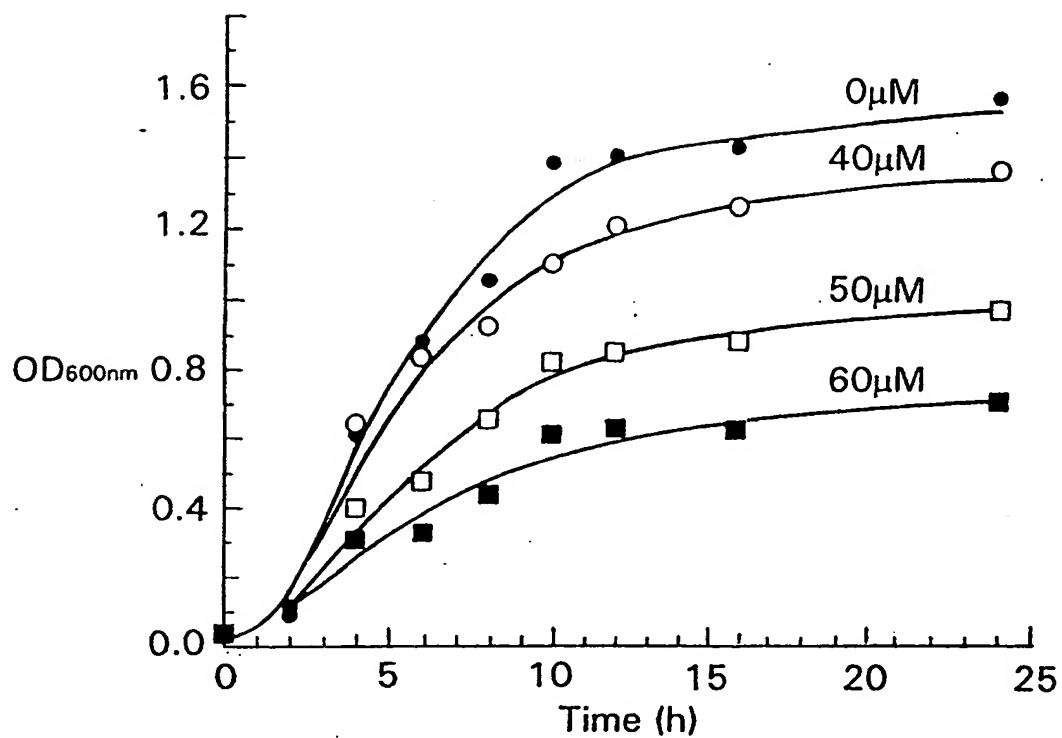
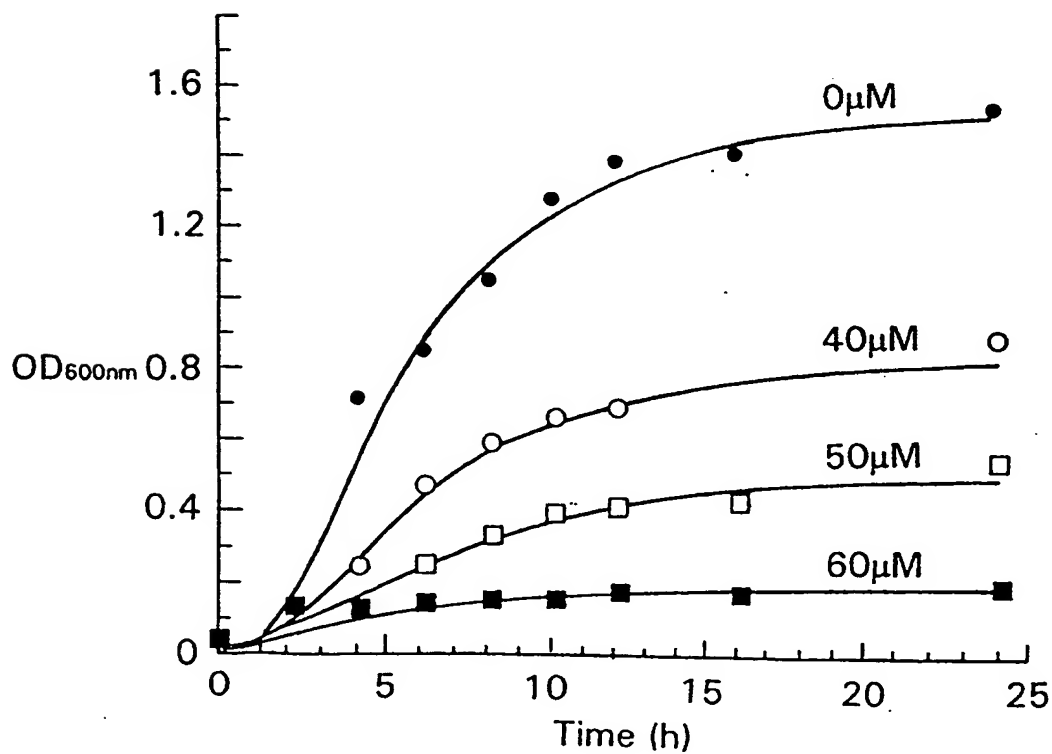


Fig. 6B



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9/41

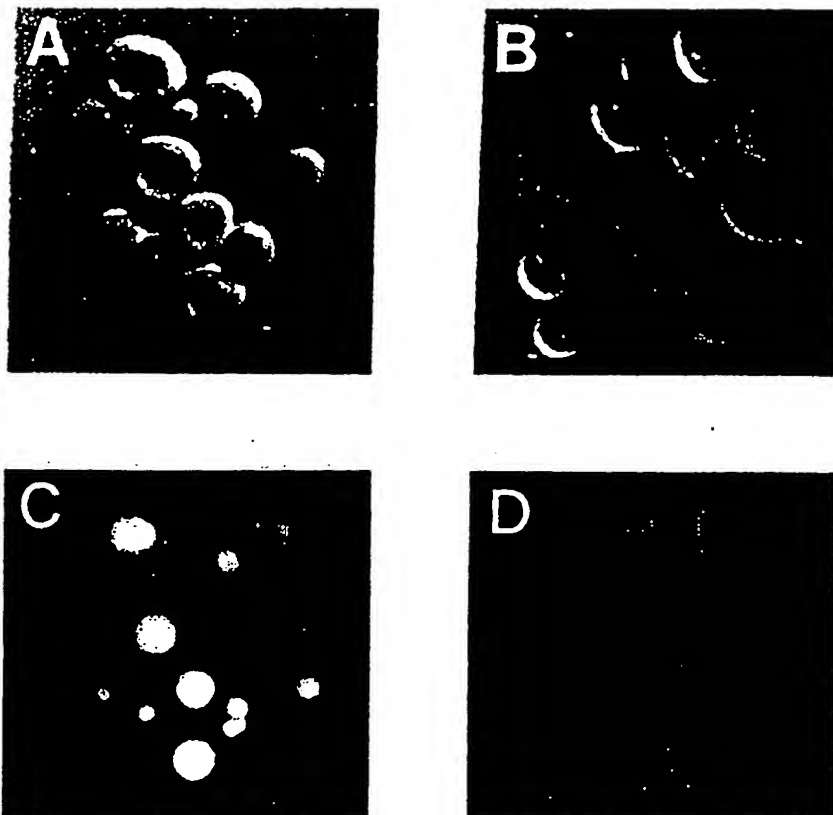
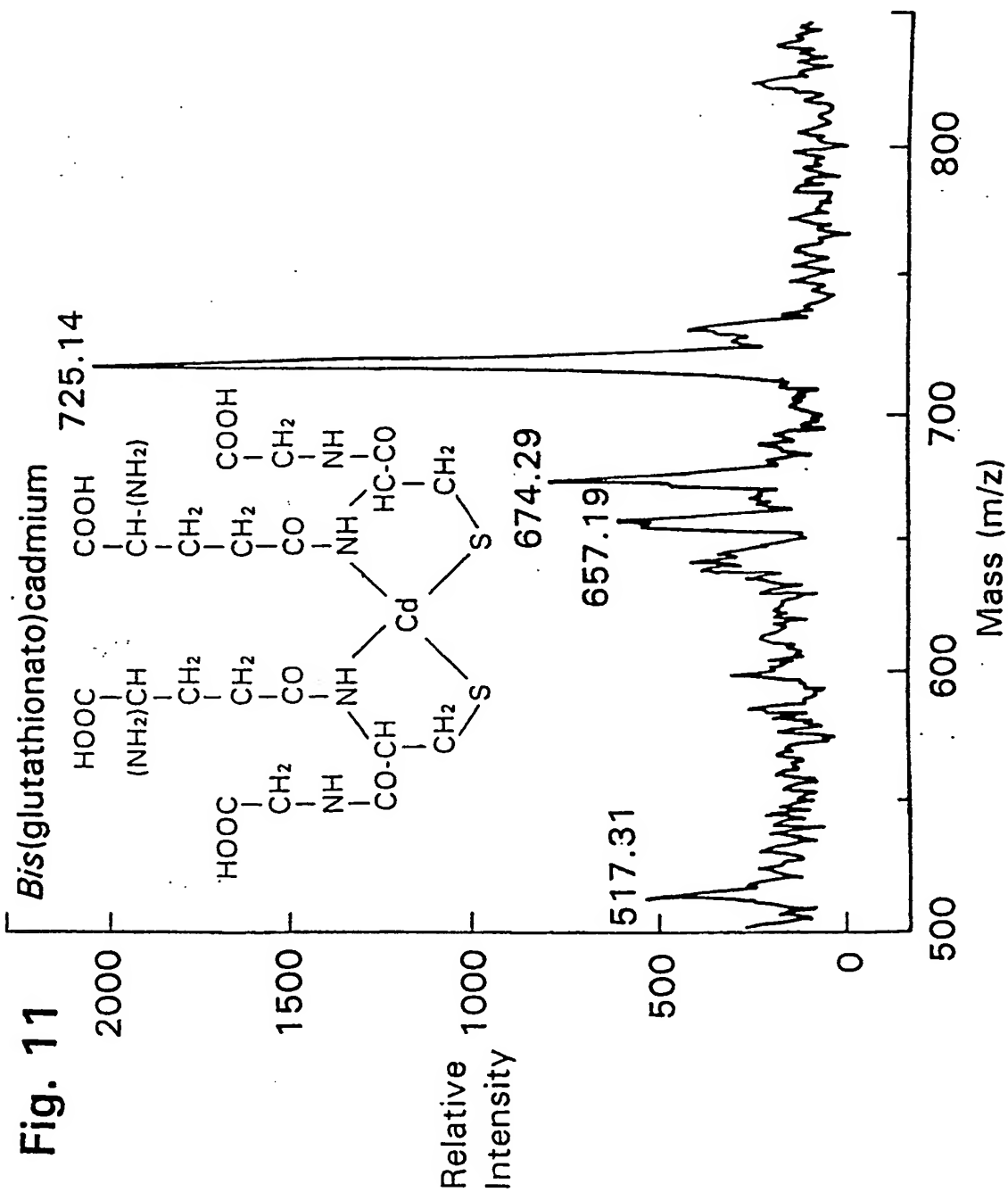


Fig. 7

16/41



17/41

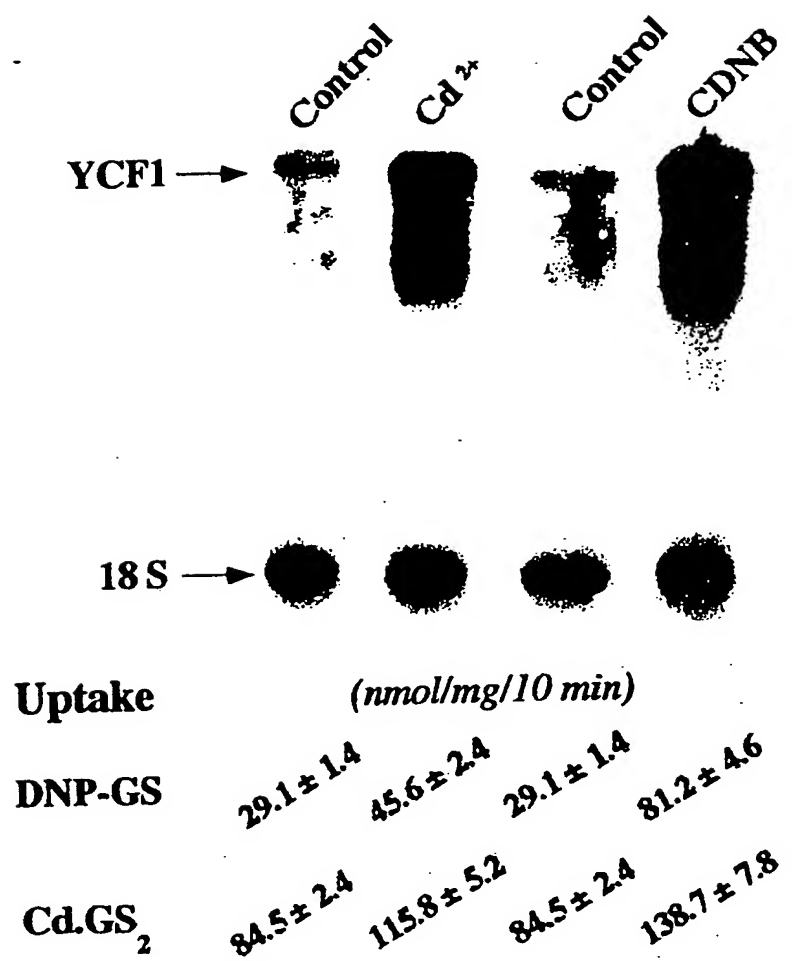


Fig. 12

ttatgaaaatttattatTTTTgttgctatggTTTTtggattagaagctcatttcaaag  
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agggaaaggaaaggataaacgATGGGGTTTGAGTTTATTGAATGGTATTGTAAGCCGGTGC  
CTAATGGTGTGTGGACTAAACAGTGGCTAATGCATTTGGTGCATACACGCCTTGTGCTA  
CTGACTCTTTTGTGCTTGGTATCTCTCAACTGGTTCTGTTGGTTCTGTGCCTGTATCGTA  
TATGGCTCGCCTTAAAGGATCACAAGGTGGAGAGGTTCTGTTTGGAGTCGAGATTGTATA  
ACTATTTCTGCTTGTGGCTGGTATGCTACTGCTGAGCCTTTGTTTAGATTGATCAT  
GGGGATTTGAGTTTGTAGATTTTGTATGGACCTGGACTTCCTCCTTTGAGGCATTGCGATT  
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TATGGTCTTGTAAATCTTGTCTCTCAGTCAAGGAGTACTATAGCAGTTATGTTCTGTA  
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TGACAAAATCTTCTCTCATGGATGAATCCCTTGATGACTTTGGGATCTAAAAGGCCTCT  
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GCTATGTGAAGCTCAATATTTCCAGAATGTGATGCGTGTGGTTACCGACTGAGATCTGC  
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AGAAAGAGGTGTTAATATCAGTGGAGGACAGAAGCAGAGGGTTTCCATGGCTAGGGCCGT  
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TGTTACAAACCAGCTCCACTTCTTATCACAAGTGGACAGAATTGTACTTGTGCATGAAGG  
CACAGTGAAAGAGGAAGGAACATATGAAGAGCTATCCAGTAATGGCCCTTTGTTCCAGAG  
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AGATCAAACAGCGGAACAACAGTTGCGAATGGGAACACAAATGGTCTTCAAATGGATGG  
AAGTGACGATAAAAAATCCAAAGAAGGAAATAAAAAAGGAGGGAAATCTGTCTCATCAA  
GCAAGAAGAACGTGAAACCGGAGTTGTAAGTTGGAGAGTCTGAAGAGGTACCAGGATGC

FIGURE 13A

ACTTGGAGGGGCATGGGTAGTGATGATGCTCCTTTTATGTTACGTCTTAACAGAAGTATT  
TCGGGTTACTAGCAGCACGTGGTTGAGTGAGTGGAAGTGCAGGAAGTCCAAAGAGTCA  
TGGACCCCTTTTCTACAATCTCATATATGCACTTCTCTCGTTTGGACAGGTTTGGTGAC  
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TGTC AACATGGGTGCCAATCGGTGGCTTGAATCCGTTTAGAACTCTGGGTGGTCTTAT  
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GCGTGCCAAAGATGACTCACACCACTTACAAGGCCAAAGGAAATGGCTGGCTTCTTCTCG  
CTGGGCTGCAGCCGCTCAGTTTGCTCTGGCTGCGAGTCTTACTTCGTGCGACAACGATCT  
TCAAAGCCTTGAAATTGAAGATGACAGCAGCATTTTGAAGAGAACAAACGATGCAGTTGT  
GACTCTGCGCAGTGTTCTCGAGGGGAAACACGACAAAGAGATTGCAGAGTCGCTTGAGGA  
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tctcttccgtaagaaacatatatttatcttaacaaaattattagtttggtttccatttc  
ataaacttaattttcacctgcaaagaaaatcaaacctgttggttcttcgtgataagta  
gagaaattacttgagtatccttctaactcaaaaaaaaaaaaaaaaaaaaaaaaaaaaa  
aaaaaaaaaaaaa

FIGURE 13B

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gaaaggataacgATGGGGTTTGAGTTTATTGAATGGTATTGTAAGCCGGTGCCTAATGGT  
GTGTGGACTAAAACAAGTGGCTAATGCATTTGGTGCATACACGCCTTGTGCTACTGACTC  
TTTTGTGCTTGGTATCTCTCAACTGGTCTGTGTTGGTCTGTGCCTGTATCGTATATGGCT  
CGCCTTAAAGGATCACAAGGTGGAGAGGTTCTGTTGAGGTGAGATTGTATAACTATTT  
CCTGGCTTTGTTGGCTGCGTATGCTACTGCTGAGCCTTTGTTTAGATTGATCATGGGGAT  
TTCAGTTTTAGATTTTGATGGACCTGGACTTCCTCCTTTTGAGgtgctttattttctgtt  
ccttattctttatcttttagtttgttggtgatgttttacctgaaacatgctattgtttgt  
gtgatttctttggcagGCATTCGGATTGGGTGTCAAAGCTTTTGCTTGGGGCGCTGTAAT  
GGTCATGATTTTAAATGGAACTAAAATTTACATCCGTGAACTCCGTTGGTATGTCAGGTT  
TGCTGTCATATATGCTCTTGTGGGGGATATGGTCTTGTAAATCTTGTCTCTCAGTCAA  
GGAGTACTATAGCAGgttggtacaattttggagttactttgggtttattgaagtcattgtt  
cttcttctacaggggtgaattcatgttttggtttctattgcagTTATGTTCTGTATCTCTAC  
ACAAGCGAAGTGGGAGCTCAGgttagctcacttggactccttttagagagtcagaatcct  
agcatgtgctatgattataaatcagaatccgatacagtttggtttctaacatcttaagag  
ggtgaattttggtttacttcagGTTCTGTTTGGAAATCTCTTGTATTATGCATCTTCCCA  
ATTTGGATACTTACCCTGGCTACATGCCAGTGCGGAGTGAACTGTGGATGATTATGAGT  
ATGAAGAGATTTCTGATGGACAACAAATATGCCCTGAGAAGCATCCAAATATATTTGACA  
gtaagtcactctacatgattttcatttgggtcgctgggtgaaacttataattagtaatca  
taatttgcaaacatcgctctctgacttttgggtcagattgatcatggggatttaggttttga  
aatttcacctgatttcttcttccaatttcttgggttgggtcacagAAATCTTCTCTCAT  
GGATGAATCCCTTGATGACTTTGGGATCTAAAAGGCCCTTAACAGAGAAGGATGTGTGGT  
ATCTAGACACTTGGGATCAGACTGAACTCTGTTACAGAGgtacttctaacaataattat  
atctcttaaaatgtatattactgaattgggtatttgatattttctgtatccttttttagTT  
TCCAGCATTCTTGGGATAAGGAACTACAAAAGCCGCAACCGTGGCTGTTGAGAGCATTGA  
ACAATAGCCTGGGAGGAAGgtagatagattttctcaccttatcggtgctgtgttctcatct  
cttttgagttttgagtagatttagatagtgctggatttctcactgtgatgtgcagatgttta  
agtgatctcttgaaagaaccatcaggtttttagaatgtgttaggaagcaagatcagaatat  
ttctacttatttaagtgttagttgtttgctatagcagcttaacacatttccatcttatcat  
aggcaatcatgcttgcttctgtactcttataaatttaagaataggggatacaactttta  
ctgtagattgggttaaatatgttttttttcttgggttcatattgcttaagcattatttctgt  
ttgttaactacatgtcgtatggggatctaattttttgaattttgtagGTTTTGGTGGGGA  
GGATTTTGGAAAGgtattttctgtctacctctttctcttttattcgtgcttccagagtcctt  
cctctcttttattcatatgatcacaggttctgcgtcatgttggataaccttctgtcacgt  
ggaagtcattttataatttacatgggtgttacagattattagaaggaaactagtgggttctta  
gtttttctttatcaattcattgtacttgaacatatttatttacatttgatgcacagATC  
GGGAATGATTGCTCACAGTTTGTGGGACCTCTTTTACTGAATCAACTCTTAAAGgtttgt  
tcttttcttggcagatttcggaaacctattattgggttcaatattcttatctgacaatatct  
ctcattttggatgtcaaactatatacagTCAATGCAAGAGGATGCGCCAGCTTGGATGGG  
TTACATCTATGCGTTCTCAATCTTTGGTGGAGTGgtatgaaatgaagtcctctttctctc  
tctctctctgtctatttggactctctctatcaacttgtgaaactgacacttgttataact  
tctgtatgtttgggtctaaggttcttctaaactgattataatagcaacactagatgtcccc

FIGURE 14A

taatgccactttttgattttgttgctcttggttttttgcgtctggttagataggttctga  
ctttatctagtgtagggtgatacttaagctacaaactcatcgagtgactgatgttgatg  
acaacgtttctagGTGTTCCGGGTGCTATGTGAAGCTCAATATTTCCAGAATGTCATGCG  
TGTTGGTTACCGACTGAGATCTGCTCTGgtaaattttaaatttgctaccctgacgttctt  
cctttgccatagtttttggtgcagatatgtttgctgatagcatgattcccagtatcttg  
tataggaataagtatatcaacatggtttcttatcctctatatatgatgcataaataagc  
cttggtgccaaaagttaggaataagtttggttgcttcagatgattgagtatgctgtttt  
tatttctggaaatttccaccatttccagatcctttcactagagaaatacaaatttagctg  
tatttctgattcagttcatcgttttctgcgtttgtagtgagtgaaattagcttgtaag  
aaatggaagatattttgaacacagatgatttttaaaattgggtcttctggtgatgactgt  
tttttttttagATTGCTGCTGTGTTCCGCAAATCGTTGAGGTTAACTAATGAAGGTCGTA  
GAAAGTTTCAAACAGGAAGATAACCAACTTAATGACGACTGATGCCGAATCTCTTCAGg  
tgagtatccctttcatattttcgaattcaagtttgcagtttctctatatcatagttgca  
gggtctgtaacatccggatcttgaatatatttttgtcgcagctgggtattgagtgggt  
tacagttactttttatgttcggttaataagaagttggatttacttagaaatgatttccagca  
tactgatctactgaatctgtttgttaggtctaagattggctatgaatagtgattgcattt  
tcatttctagctagcactttgttatcattgaatttttcttctctctttttattttgttt  
cttatgccaaacttaaactgtgtcttgtttaatgttttgcgtcttaactgtgtctggatca  
atattgttatctaatcaaccagatgtactttgtactaatttttccattttctgtggcagC  
AAATATGCCAATCACTTCATACCATGTGGTCCGCTCCATTTTCGTATAATTATAGCACTGA  
TTCTCCTCTATCAGCAATTGGGTGTTGCCCTCGCTCATTGGTGCATTGTTGTTGGTCCTTA  
TGTTCCCTTTACAGgtacatgacttctaaatttctcatttttttcttctgtagcttat  
ttttctctatactgttcgcttgttcattcgtaactctaaaggctacttcttctctgctctc  
ctgaacttgttctctgttttcttaaacagACTGTTATTATAAGCAAAATGCAGAAGCTG  
ACAAAGGAAGGTCTGCAGCGTACTGACAAGAGAATTGGCCTTATGAATGAAGTTTtagCT  
GCAATGGATACAGTAAAgtaagaaattctagaaccaattttgttaacatagttattaatt  
tgcaggaaacttgtactaaaccaaagtgtacagGTGTTATGCTTGGGAAAACAGTTTCC  
AGTCCAAGGTCCAACTGTCGTGATGATGAATTATCTTGGTTCCGGAATCACAGCTCCT  
GGGAGCGgtatgactacagcgtagttacttttgttttctccttaattattgtatatttct  
aactcttgccttggcttctgttcttgggttgcagTTGAATATGTTTCACTGAATAGCATTCC  
TGTTCTTGCTGACTATTGTTTTCATTTGGTGTGTTTACATTACTTGGAGGAGACCTGACCCC  
TGCAAGAGCATTTACGTCACCTCTCTCTCTTTGCTGTGCTTCGTTTCCCTCTCTTCATGCT  
TCCAAACATTATAACTCAGgtgatttcttaaatatgttggttgcaatgcatgtgattaag  
tagaactgttagtgctttagtaactgtcggtttgggttatcaaatccatgacttatatttc  
gaatttacatgctggagggtatccttgctgggtgccagaaacagatgccgatgctgactag  
ttttcacttgtagGTGGTAAATGCTAATGTATCCTTAAAACGTCTTGAGGAGGTATTGGC  
GACAGAAGAAAGAATTCTCTTACCAAATCCTCCCATTGAACCTGGAGAGCCAGCCATCTC  
AATAAGAAATGGATATTTCTCTTGGGATTCTAAGgtgtcgcttggctattctataccatg  
ttccttcttctcgcttctctcattacctttatccatagaaagtacaaaaatcgagctaacc  
ctatgtatctacagGGGATAGGCCGACGTTGTCAAATATCAACTTGGATGTACCTCTTG  
GCAGCCTAGTTGCTGTGGTTGGTAGTACAGGCGAAGGAAAAACCTCTCTAATATCTGCTA  
TCCTTGGTGAACCTTCTGCAACATCTGATGCAATAGTTACTCTCAGAGGATCAGTTGCTT  
ATGTTCCACAAGTTTCATGGATCTTTAATGCAACAgatgttcttcttcttcttctgacttt  
taagtgggctgacgttgcaaattttctgttgtagcataatgttaaatgtattttctgtc  
accttcttcaatatttgattctctaaagctattttgaacagaagactgatatgcattttct  
tataaaaattttagGTACGCGACAATATACTGTTTGGTTCTCCTTTTCGACCGTGAAAAG  
TATGAAAGGGCCATTGATGTGACTTCACTGAAGCATGACCTAGAGTTACTGCCTgtaagt  
tttgaggagagcttcgtggaggttgataacaaggatttgcgttgccgtgttctcggtgtgct

FIGURE 14B

aagtttgtttcaacctctttctcttgcttaatagGGTGGTGATCTCACGGAGATTGGAGA  
AAGAGGTGTTAATATCAGTGGAGGACAGAAGCAGAGGGTTCCATGGCTAGGGCCGTTTA  
CTCAAATTCAGATGTGTACATCTTTGATGACCCGTTAAGTGCCCTTGATGCTCATGTTGG  
TCAACAGgtactaactcattgattctcttttgataaggctagtctatttcatttttgaatt  
tatctaacattttttgtgtctggtcattatgggaataactgtcagtctgatttcttaggaata  
ttgtttcagGTTTTTGAAAAATGCATAAAAAGAGAACTGGGGCAGAAAACGAGAGTTCTT  
GTTACAAACCAGCTCCACTTCCTATCACAAGTGGACAGAATTGTACTTGTGCATGAAGGC  
ACAGTGAAAGAGGAAGGAACATATGAAGAGCTATCCAGTAATGGGCCCTTTGTTCCAGAGG  
GTAATGGAAAATGCAGGGGAAGGTGGAAGAATATTCAGAAGAAAATGGAGAAGCTGAGGCCA  
GACCAAACAGCGGAACAACCAGTTGCGAATGGGAACACAAATGGTCTTCAAATGGATGGA  
AGTGACGATAAAAAATCCAAAGAAGGAAATAAAAAAGGAGGGAAATCTGTCCTCATCAAG  
CAAGAAGAACGTGAAACCGGAGTTGTAAGTTGGAGAGTCCTGAAGAGgtaacttgaacat  
ttggccttttgcaactcttactatttgtttgcaactttccccatactcgatccaagaggtcc  
attcatttgtggtgtttcacaacaaactagcatgttccttatgttttttaggctgaactat  
acctttgcgggatatacagaatgacttttccaggctttcaatgttttcagGTACCAGGATG  
CACTTGGAGGGGCATGGGTAGTGATGATGCTCCTTTTATGTTACGTCTTAACAGAAGTAT  
TTCGGGTTACTAGCAGCACGTGGTTGAGTGAGTGGACTGATGCAGGAACTCCAAAGAGTC  
ATGGACCCCTTTTCTACAATCTCATATATGCACTTCTCTCGTTTGGACAGgtatgagtta  
tgtttgcttgatggatgagtgaagatttgatataatcttgacctcatgatataacatata  
tagctgaaacctgaccagcttagaaagatcttatataattctacttttgtgattttactt  
tgagaatccaaaggtggaggtagaaaaggttagtaaagaattgatttttttgtctgagact  
ctttctctcttgcttacagGTTTTGGTGACATTGACCAATTCATATTGGTTGATTATGTCC  
AGTCTTTATGCAGCTAAGAAGTTACACGACAATATGCTTCATTCCATACTGAGGGCCCCG  
ATGTCCTTCTTCCATACCAATCCGCTAGGACGGATAATCAATCGATTTCGCAAAAGATCTG  
GGTGATATTGATCGAACTGTGGCCGCTCTTTGTAAACATGTTTATGGGTCAAGTCTCACAG  
CTTCTTTCAACTGTAGTGTGATTGGCATTGTAAGCACTTTGTCTTGTGGGCCATCATG  
CCCCTCCTGGTCTTGTTTTATGGAGCTTATCTTTATTATCAGgtaatgtaccttctgacc  
gcagcattttaataaactgagattaagtacagaaagagaaaaggacacagatgatggatg  
ttacacatacttttttagcctcatttgtcatgtctgagttcgtttggtgcttaagctatc  
tacactcatctgtccacaaaaatcatgctgtatatgttggtgtgttaaatatttttcttat  
tgcagAACACAGCCCGTGAGGTTAAGCGTATGGATTCAATTTCAAGATCGCCTGTTTATG  
CACAGTTTGGAGAGGCATTGAATGGCTTATCAACTATCCGTGCTTACAAAGCATATGATC  
GTATGGCTGATATCAACGGAAGATCAATGGATAATAACATCAGATTCACTCTTGTCAACA  
TGGGTGCCAATCCGGTGGCTTGAATCCGTTTAGAAACTCTGGGTGGTCTTATGATATGGC  
TGACAGCATCGTTTGTCTGTATGCAGAAATGGAAGAGCGGAGAACCAACAGGCATTGTCAT  
CTACAATGGGTTTGTCTCTCAGTTATGCCTTAAATATTACTAGCTTGTTAACAGGTGTTT  
TGAGACTTGCGAGTTTGGCTGAGAAATAGTCTAAACGCGGTGAGTGTGGCAATTATATA  
GAGATTCCGCCAGAGGTCCGCCTGTCATTGAGAACAACCGTCCACCTCCTGGATGGCCAT  
CATCTGGATCCATAAAGTTTGAGGATGTTGTTCTCCGTTACCGCCCTCAGTTACCGCCTG  
TGCTTCATGGGGTTTCTTTCTTCATTCATCCAACAGATAAGGTGGGGATTGTTGGAAGGA  
CTGGTGCTGGAAAGTCAAGCCTGTTGAATGCATTGTTTAGAATTGTGGAGGTGGAAAAAG  
GAAGGATCTTAATCGATGATTGTGACGTTGGAAAGTTTGGACTGATGGACCTACGTAAAG  
TGCTCGGAATCATTCCACAGTCAACGGTCTTTTCTCAGGAAGTGTGAGGTTCAATCTTG  
ATCCATTTGGTGAACACAATGATGCTGATCTTTGGGAATCTCTAGAGAGGGCACACTTGA  
AGGATACCATCCGCAGAAATCCTCTTGGTCTTGATGCTGAGgtattcagttgctgcctat  
attgatatgaagtctcattttttaagtggtaataactgattttcaatctttgttcagGTC  
TCTGAGGCAGGAGAGAATTTAGCGTGGGACAGAGGCAATTGTTGAGTCTTTACGTGCG  
CTGTTACGGAGATCTAAGATACTCGTCCTTGATGAAGCAACTGCTGCTGTAGATGTTAGA  
ACCGATGCCCTCATTGAGAAGACTATCCGAGAAGAATTCAAGTCATGCACGATGCTCATT

FIGURE 14C



ATCGCTCACCGTCTCAATACCATCATTGACTGTGACAAAATTCTCGTGCTTGATTCTGGA  
AGAgatgatgttttaaacactctctctctttcaatctcacactctccttggtttctcagcta  
acctgttctattccaatttggttaactcagGTTCAAGAATTCAGTTCACCGGAGAACCTTC  
TTTCAAATGAAGGAAGCTCTTTCTCCAAGATGGTTCAAAGCACTGGAGCTGCAAATGCTG  
AGTACTTGCGTAGTTTAGTACTCGACAACAAGCGTGCCAAAGATGACTCACACCACTTAC  
AAGGCCAAAGGAAATGGCTGGCTTCTTCTCGCTGGGCTGCAGCCGCTCAGTTTGCTCTGG  
CTGCGAGTCTTACTTCGTGCGACAACGATCTTCAAAGCCTTGAAATTGAAGATGACAGCA  
GCATTTTGAAGAGAACAAACGATGCAGTTGTGACTCTGCGCAGTGTTCTCGAGGGGAAAC  
ACGACAAAGAGATTGCAGAGTCGCTTGAGGAACATAATATCTCTAGAGAGGGATGGTTGT  
CATCACTCTATAGAATGGTAGAAGgtaaaccaaataatgcatctctacaaatgcttatgca  
aaatcttaatcaccacactgaaacattaaagtcaaatacgtgctcttatattgcaagcctg  
ctttccgctgtctacgtttcagGGCTTGCAAGTATGAGCAGATTGGCAAGGAACCGAATG  
CAACAACCGGATTACAATTCGAAGGAAATACATTTGACTGGGACAACGTCGAGATGTAG  
ATAAGTTTCATGTTAACTAGGAATCATTGTCTCTTCCGTAAGAAACATATATTTATCTTA  
ACCAAAATTATTAGTTTGGTTTCCATTTTATAAACTTAATTTTACCTGCAAAGAAAATC  
AAACCCTGTTGTGTTCTTCGTGATAAGTAGAGAAATTACTTGAGTATCCTTCTAACTCat  
aatgggatctcatgattcatgaacaagcagcaacacaataatacccttttcagattttg  
gagctggacaaagttgttaagttgagtttctcttacagtcattcatatacaaaaacctct  
tcgactgaagcaccaagaaagaaacaaacatcaaaagggaatgaggtcttttcttagggc  
tgagatcatcggaatgtgggagtgcggaacacgacc

FIGURE 14D

MGFEFIEWYCKPVPNGVWTKTVANAFGAYTPCATDSFVLGISQLVLLVLCYRIWLALKD  
HKVERFCLRSRLYNYFLALLAAYATAEPLFRLIMGISVLDDFDGPGLPPFEAFGLGVKAF  
WGAVMVMILMETKIYIRELRWYVRFAYIYALVGDMLNVLVLSVKEYYSSYVLYLYTSEV  
GAQVLFGILLFMHLPNLDTPGYMPVRSETVDDYEYEEISDGQQICPEKHPNIFDKIFFS  
WMNPLMTLGSKRPLTEKDVWYLDTWDTETLFTSFQHSWDKELQKPQPWLLRALNNSLGG  
RFWWGGFWKIGNDCSQFVGPLLLNQLLKSMQEDAPAWMGYIYAFSIFGGVVFVGLCEAQY  
FQNMVRVGYRLRSALIAAVFRKSLRLTNEGRRKFQTKITNLMTTDAESLQICQSLHTM  
WSAPFRIIIALILLYQQLGVASLIGALLLVLMFPLQTVIISKMQKLTKEGLQRTDKRIGL  
MNEVLAAMDTVKCYAWENSFQSKVQTVRDELSWFRKSQLLGALNMFILNSIPVLVTIVS  
FGVFTLLGGDLTPARAFTSLSLFAVLRFPFLMPLNIITQVVNANVSLKRLEEV LATEERI  
LLPNPIEPGEPAISIRNGYFSWDSKGRPTLSNINLDVPLGSLVAVVGSTGEGKTSLSIS  
AILGELPATSDAIVTLRGSVAYVPQVSWIFNATVRDNILFGSPFDREKYERAIDVTS LKH  
DLELLPGGDLTEIGERGVNISGGQKQRVSMARAVYSNSDVYIFDDPLSALDAHVGQQVFE  
KCIKRELQKTRVLVTNQLHFLSQVDRIVLVHEGTVKEEGTYEELSSNGPLFQRLMENAG  
KVEEYSEENGAEADQTAEQPVANGNTNGLQMDGSDDKKSKEGNKKGGKSVLIKQEERET  
GVVSWRVLKRYQDALGGAWVVMMLLLCYVLTEVFRVTSSTWLSEWTDAGTPKSHGPLFY  
LIYALLSFGQVLVTLTNSYWLIMSSLYAAKKLHDNMLHSILRAPMSFFHTNPLGRIINRF  
AKDLGDI DRTVAVFVNMFMGQVSQLLSTVVLIGIVSTLSLWAIMPLLVLFYGAYLYYQNT  
AREVKRMDSISRSPVYAQFGEALNGLSTIRAYKAYDRMADINGRSMDNNIRFTLVNMGAN  
RWLGIRLETLGGLMIWLTASFVQMONGRAENQQAFAS TMGLLSYALNITSLLTGVLRLA  
SLAENSLNAVERVGN YIEIPPEAPPVIENNRPPPGWPSSSGSIKFEDVVLRYRQPLPPVLH  
GVSFFIHPTDKVGIVGRTGAGKSSLLNALFRIVEVEEGRILIDDCDVGKFGMLDLRKVLG  
IIPQSPVLFSGTVRFNLDPFGEHNDADLWESLER AHLKDTIRRNPLGLDAEVSEAGENFS  
VGQRQLLSLSRALLRRSKILVLDEATAAVDVRTDALIQKTIREEFKSC TMLIIAHLNNTI  
IDCDKILVLDSGRVQEFSSPENLLSNEGSSFSKMVQSTGAANA EYLRSLVLDNKRKDDSD  
HHLQGQRKWASSRWAAAAQFALAASLTSSHNDLQSLEIEDDSSILKRTND AVVTLRSVLE  
GKHDKEAESLEEHNISREGWLSSLYRMVEGLAVMSRLARNRMQQPDYNFEGNTFDWDNVE  
M

FIGURE 15

25/41

gaattcgcgccgcccgcgaatttgcaactctttacctctctttgactccgtgagattcga  
ggattgttagtttcttgtgatgtgtagtctttgaagcaggggatttttattgtattgagg  
aagaagATGGGGTTTGAGCCGTTGGATTGGTATTGCAAGCCGGTGCCGAATGGTGTGTGG  
ACTAAACTGTGGATTATGCGTTTGGTGCATACACGCCTTGTGCTATTGACTCTTTTGTG  
CTTGGTATCTCTCATCTGGTTCTGTTGATTCTGTGTCTTTATCGCTTGTGGCTCATCACG  
AAGGATCACAAAGTGGATAAGTTCTGCTTGAGGTCTAAATGGTTTAGCTATTTTCTGGCT  
CTTTTGGCTGCTTATGCTACTGCGGAGCCTTTGTTTAGATTGGTCATGAGGATCTCTGTT  
TTGGATTGGATGGAGCTGGGTTTCCTCCCTATGAGGCGTTTATGTTGGTCCCTTGAGGCT  
TTTGCTTGGGGTTCTGCTTTGGTCATGACTGTTGTGGAAACTAAAACGTATATCCATGAA  
CTCCGTTGGTATGTGAGATTGCTGTCTATTATGCTCTTGTGGGAGACATGGTGTGTGA  
AATCTTGTCTCTCTGTTAAGGAGTACTATGGCAGTTTAAACTGTATCTTTACATAAGC  
GAGGTGGCAGTTTCAAGTTGCATTTGGAACCCTCTGTTTGTGTATTTCCCTAATTTGGAC  
CCTTACCCTGGTTACACACCAGTTGGGACTGAAAATTCCGAGGATTACGAGTATGAAGAG  
CTTCTGAGGAGAGAAAATATATGTCTGAGAGGCATGCAAATTTATTTGACAGTATCTTC  
TTCTCATGGTTGAACCCATTGATGACTCTGGGATCAAAACGACCTCTCACCGAGAAGGAT  
GTATGGCATCTGGACACTTGGGATAAACTGAACTCTTATGAGGAGCTTCCAGAAGTCC  
TGGGATAAGGAACTAGAAAAGCCCAAACCGTGGCTTTTGAGAGCACTGAACAACAGCCTT  
GGGGGAAGGTTTCTGTTGGGTTGGCTTTTGGGAAGATTGGGAATGACTGTTACAGTTCTGTG  
GGGCTCTTCTACTGAATGAGCTCTTAAAGTCAATGCAACTTAATGAACCAGCGTGGATA  
GGTTACATCTATGCAATCTCAATCTTTGTTGGAGTGGTATTGGGGGTTTATGTGAAGCT  
CAGTATTTCCAAAATGTGATGCGTGTGGTTACCGGCTTAGGTCTGCACTGATTGCTGCT  
GTGTTCCGAAAATCTTTGAGGCTAACTAATGAGGGCGGAAGAAGTTTCAAACAGGAAAA  
ATAACAACTTAATGACTACTGATGCTGAGTCTGCTGAGCAAATCTGCCAATCACTTCAT  
ACCATGTGGTCCGCGCCATTTCTGATAATTGTAGCACTGGTCTCTCTCTATCAACAATTG  
GGTGTGCTCGATCATTTGGTGCATTTGTTTCTTGTCTTATGTTCCCATACAGACTGTT  
ATTATAAGCAAAACGCAGAAGTTAACAAAAGAAGGGTTGCAGCGTACTGACAAGAGAATT  
GGCCTAATGAATGAGGTTTGTAGCGCAATGGATACAGTGAAGTGTACGCTTGGGAAAAC  
AGTTTTTCAGTCCAAGGTTCAAACGTGACGTGATGATGAATTATCTTGGTTCCGGAAGCA  
CAACTCCTGTGAGCGTTCAATATGTTTCACTATAACAGCATCCCTGTCTCTGACTGTT  
GTTTCATTTGGTGTGTTCTCATTGCTTGGAGGAGATCTGACACCTGCAAGAGCGTTTACG  
TCACTCTCTCTATTTTCTGTGCTTCTGCTTCCCTTATTTCATGCTTCCAAACATTATAACT  
CAGATGGTAAATGCTAATGTATCCTAAACCGTTTGGAGGAGGTACTGTCAACCGAAGAGA  
GAGTTCTCTTACCGAATCCTCCCATTTGAACCTGGACAGCCAGCTATCTCAATAAGAAATG  
GATACTTCTCCTGGGATTCAAAGGCGGATAGGCCAACATTGTCAAACATCAACCTGGACA  
TACCTCTTGGCAGCCTAGTTGCGGTAGTTGGCAGCACAGGAGAAGGAAAAACCTCCCTGA  
TATCTGCTATGCTTGGGGAACCTTCTGCAAGATCTGATGCGACTGTTACTCTTAGAGGAT  
CAGTCGCTTATGTTCCACAAGTTTTCATGGATCTTTAACGCAACAGTACGTGACAAATATAT  
TGTTTGGGGCTCCTTTTGACCAAGAAAAATATGAAAGGGTGATTGATGTGACAGCACTCC  
AGCATGACCTTGAGTTACTGCTTGGAGGTGACCTCACGGAGATCGGAGAAAGGGGTGTTA  
ACATCAGTGGGGGACAAAAGCAGAGGGTTTCTATGGCTAGGGCCGTTTACTCAAATTCAG  
ACGTGTGCATCTTAGATGAACCATTGAGTGCCCTTGATGCGCATGTTGGTCAGCAGGTTT  
TTGAAAAATGCATAAAAAGGGAAGTGGGAGACAAACGAGAGTACTTGTACAAATCAGC  
TCCACTTCTATCACAGTGGATAAAATCCTACTTGTCCATGAGGGAACAGTAAAAGAGG  
AAGGAACATATGAAGAATTATGCCATAGTGGCCCGTTGTTCCCGAGGTTAATGGAAAATG  
CAGGGAAGGTTGAAGATTATCCGAAGAAAATGGAGAAGCTGAAGTACATCAAACATCTG  
TAAACAGTTGAAAATGGGAACGCTAATAATCTGCAGAAGGATGGAATCGAGACAAAGA  
ATTCCAAAGAAGGAACTCTGTTCTTGTCAAACGAGAAGAACGTGAACTGGAGTTGTGA  
GTTGGAAAGTCTTGGAGAGGTACCAGAATGCACTTGGAGGTGCATGGGTAGTGATGATGC

FIGURE 16A

TCGTTATATGCTACGTCTTGACTCAAGTATTTCTGGGTTTCAAGCATCACTTGGTTGAGTG  
AGTGGACTGATTCAGGAACCCCAAAGACTCATGGACCCCTATTCTATAATATTGTCTATG  
CGCTTCTTTTCGTTTGGACAGGTCTCTGTGACATTGATCAATTCATATTGGTTGATTATGT  
CCAGTCTATATGCAGCTAAAAAGATGCATGATGCTATGCTTGGTTCCATACTAAGGGCTC  
CAATGGTGTCTTTCAAACCAATCCATTAGGACGGATAATCAATCGATTTGCAAAAGATA  
TGGGAGATATTGATCGAACTGTGGCAGTCTTTGTAAACATGTTTATGGGTTCAATCGCAC  
AGCTTCTTTCAACTGTTATCTTGATTGGCATTGTGAGCACTCTGTCCCTGTGGGCCATCA  
TGCCCCCTGTTGGTCGTGTTCTATGGAGCTTATCTGTATTACCAGAACACATCTCGGGAAA  
TTAAACGTATGGATTCCACTACAAGATCGCCAGTTTATGCTCAATTTGGTGAGGCATTGA  
ATGGACTATCTAGTATCCGTGCTTATAAAGCATATGACAGGATGGCTGAAATTAATGGAA  
GGTCAATGGACAATAACATCAGATTACACTTGTAAACATGGCTGCAATCGGTGGCTGG  
GAATCCGTTTGGAAAGTTTGGGAGGTCTCATGGTTTGGTGGACTGCTTCATTAGCCGTCA  
TGCAGAACGGAAAGGCAGCGAACCAACAAGCATATGCATCTACGATGGGTTTGCTTCTCA  
GTTATGCGTTAAGCATTACCAGCTCTTTAACAGCTGTACTGAGACTCGCGAGTCTAGCTG  
AGAATAGTTTAACTCGGTTGAGCGTGTTGGAAATTATATCGAGATACCATCAGAGGCTC  
CATTGGTCATTGAAAACAACCGTCCACCTCCCGGATGGCCATCATCTGGATCCATAAAT  
TTGAGGATGTTGTTCTTCGTTACCGCCCTGAGTTACCTCCTGTTCTTCATGGAGTTTCGT  
TCTTGATTTCTCCAATGGATAAGGTGGGAATTGTTGGGAGGACAGGCGCTGGGAAATCAA  
GCCTCTTAAATGCCTTATTTCAGGATTGTGGAGCTGGAAAAAGGAAGGATTTAATTGATG  
AATGCGACATTGGAAGATTTGGACTGATGGACCTACGTAAAGTGGTCGGAATTATACCGC  
AAGCGCCAGTTCTTTTCTCAGGTACCGTGAGATTCAATCTTGACCCATTTAGTGAACACA  
ACGACGCCGATCTCTGGGAATCTCTTGAGAGGGGCACACTTGAAAGATACTATCCGCAGAA  
ATCCTCTTGGTCTTGATGCTGAGGTAAGTGAAGGAGAGAAATTTAGTGTGGACAGA  
GACAGTTGTTGAGTCTTGACGTGCATTGTTACGAAGATCTAAGATACTTGTCTTGATG  
AAGCAACTGCTGCAGTTGACGTAAGAACTGATGTTCTCATCCAAAAGACCATCCGAGAAG  
AATTCAAGTCATGCACAATGCTAATCATCGCTCATCGTCTCAATACTATCATCGACTGTG  
ACAAAGTTCTTGTCCTTGATTCTGGAAAAGTTTCAAGGAAATTCAGTTTACCGGAGAATCTTC  
TTTCAAATGGAGAAAGTTCTTCTCGAAGATGGTTCAAAGTACAGGAACTGCAACGCGG  
AGTACTTACGTAGTATAACACTAGAGAACAAACGTACCAGAGAAGCTAACGGTGATGATT  
CACAACCTTTAGAAGGTCAAAGGAAATGGCAAGCTTCTTCTCGTTGGGCTGCAGCTGCTC  
AATTTGCATTGGCTGTGAGCCCTCACTTCATCTCACAACGACCTCCAAAGCCTTGAAATCG  
AAGATGATAACAGTATTTTGAAGAAAACAAAGGACGCCGTCTCACTTTACGCAGTGTCC  
TTGAAGGGAAACATGATAAAGAGATTGAAGACTCTCTAAACCAAAGTGACATCTCTAGAG  
AGCGTTGGTGGCCATCTCTTTACAAAATGGTCTGAAGGGCTTGCCGTGATGAGCAGATTGG  
CGAGGAACAGAATGCAACACCCGGATTACAATTTAGAAGGGAAATCGTTTGACTGGGACA  
ATGTCGAGATGTAAacgatgaaaggcttacactaatagacctaaaactcccattttgatg  
gaacttttattttgtattgcttgggatacacgtaacaaaatgcccattaatcggtggtgtaa  
ctatataggctatgcttcttttgggaaaaagagagtttgattacagaggatgtgatgata  
acacaattggaattc

FIGURE 16B

gggaggttttggttttttccctatcaatcgaattccatttcgtgctcgtaacgtggatttttggtaga  
tttttttaggggatggaaacttggttattatctatagatgatgattttgttttctccatgagaa  
tgtatgcttttaaaactttttttttgtttttgcttcggagctaactttgggggctggtctcg  
gtctctgttttctctccactaaaaagataaaaagcctttgccatcttttttttctcaataatc  
tatcacatcggttttttttctttgttttttctccatttgcttctcattgagttcatagccacataat  
tattgatttcttttttcttttagtggttctggttactgatgcgtttcattatttatacttctcacttg  
cagattcgaggattggttagtttctgtgatgtgtagtctttgaagcaggggatttttattgtattg  
aggaagaagATGGGGTTTGAGCCGTTGGATTGGTATTGCAAGCCGGTGCCGAATGGTGTGTGGACT  
AAACTGTGGATTATGCGTTTGGTGCATACACGCCCTTGCTATTGACTCTTTTGTGCTTGGTATC  
TCTCATCTGGTTCTGTGATTCTGTGCTTTTATCGCTTGTGGCTCATCACGAAGGATCACAAAGTG  
GATAAGTTCTGCTTGAGGTCTAAATGGTTTAGCTATTTTCTGGCTCTTTTGGCTGCTTATGCTACT  
GCGGAGCCTTTGTTTAGATTGGTTCATGAGGATCTCTGTTTTGGATTGGATGGAGCTGGGTTTCCT  
CCCTATGAGgtggttatcactttgctgtttgttgatgttggttctcctctgtatgtttttcct  
gagagatgctgtgtttgtgcttttatttggcagGCGTTTATGTTGGTCCTTGAGGCTTTTGCTTG  
GGTTCGCTTTGGTTCATGACTGTTGTGGAACTAAAACGTATATCCATGAACCTCCGTTGGTATGT  
CAGATTGCTGTCAATTTATGCTCTTGTGGGAGACATGGTGTGTAAATCTTGTCTCTCTGTTAA  
GGAGTACTATGGCAGgttggttaaatttgcagtcgtatggtttatgcaattttgtttccctgggtct  
ggcagatgaacttatatgctcattttttttgttttggcagTTTTAACTGTATCTTTACAT  
AAGCGAGGTGGCAGTTTCAGgtttgcactttaaactccttttgcattctccaaactactctttac  
catgtgctgtatctaagtcacactgtaaataatgatacaactttgttttataatgacgttaaggatgg  
tttttggtaccagGTTGCATTTGGAACCTCTTGTGTTGTGTATTTCCCTAATTTGGACCTTACCC  
TGGTTACACACCACTTGGGACTGAAAATTCGAGGATTACGAGTATGAAGAGCTTCTGAGGAGA  
AAATATATGTCCTGAGAGGEATGCAAATTTATTTGACAGtatgtcactctacacttctcattcct  
actttgtttttataggtgcattttctatttttaattgtgagaattgccaccgcatcttttatcactt  
ttctgcacttactacctatctaagttggttatttatgagagcttaaataatttccctggaattgta  
aattttcttatggagtgtataacgtagtaggtcattaaaattgtttccgcagagagtagtctata  
gtctctccaaaatttttttgacttatectcccggtctccttagaaatgaacttatgatttgtga  
ctgtgccgagggttttgccttagtgatcatcacttcgactaagctgcaacattttatatagtatatt  
cgtcaacatttgtcaactttgactattatgttccctcttacccttgcttttcaaccacagGTAT  
CTTCTTCTCATGGTTGAACCCATTGATGACTCTGGGATCAAAACGACCTCTCACCGAGAAGGATGT  
ATGGCATCTGGACACTTGGGATAAAAAGTAACTCTTATGAGGAGgtatatattttaaataaatacaa  
ctgttctcatactgtctatgactggcatggttgcgtgacatatttttatctcatttttttagCTTCC  
AGAAGTCTCTGGGATAAGGAAGTGAAGAAAGCCAAACCGTGGCTTTTGAGAGCACTGAACAACAGCC  
TTGGGGGAAGgtaaacaaaaacttcttcacagtcagtgtgttttcatctttttgggctttgacatga  
tgtgtgatttgtaaaaggaagcatttgggttgtaataataaatgcattatgaataactagaagctga  
gaaatctgttatggctgtgacttcaagtatgttttgatgcgtgtcgagttgaataagaaatgtgtt  
acttttctggttataatctgccatagatactttccatccttatggactgtctgtttctgcattttg  
tagGTTTTGGTGGGGTGGCTTTTGAAGgtacttttgtActctttattgtgttttattctttattc  
tgaaacagtcttttcccttgctctatttgataatattgatggcttctgaggtcttagttttcctaaat  
ggtgtgttttgtaactgtttaactcttgacatttcaatctaaattgtatcatagATTGGGAATGACT  
GTTTCACAGTTTCGTGGGGCCTCTTCTACTGAATGAGCTCTTAAAGgtttgttcccttacttctttt  
accccggtgcacattgtgcttgaacctatttaacacaatgctttgttaatttttccattcacatggat  
ctttgagatggattcatattcctactggctcgaataagtggtttaacgttcttgatagattcaaaa  
tcctatcatcctttgaatattatgttctgacgatatctcacaatgtctcctttaactttccgcagT  
CAATGCAACTTAATGAACCAGCGTGGATAGGTTACATCTATGCAATCTCAATCTTTGTTGGAGTGg  
tatgcaacaaattctcttttctctcgctgcctttattattctcttgcatggactgcaaaggatag  
aaacaaaaactctactttccttggattcttttcttcttctgctaggacttcatggtattttgggtct  
agagtagatgctacgaattgtaggaccagtttaattttcttaagctgaaagtaatctctgtgcat  
tcgattgtattagaaaatagcctgattctactcttagagtttagtttttttggtttaatacatt  
tgcatgttgaaaagggttttgtttaatgtaggtcaagggtgacacttgaccaatggactccttgatcg  
cttgatgttgatgttgacattttcagGTATTGGGGGTTTTATGTGAAGCTCAGTATTTCAAAATG  
TGATGCGTGTGGTTACCGGCTTAGGTCTGCACTGgttaagaaaaagtttccatgaattatctttt  
gctacttagtttttctt

FIGURE 17A

SUBSTITUTE SHEET (RULE 26)

tttgctctgcttctcatgttttgatgcaataacctgtactgttatgtctgttgaaagctatagcaga  
tgcttatagattgcttcattctgctgatgaattctcccttaataagATTGCTGCTGTGTTCCGAAAA  
TCTTTGAGGCTAACTAATGAGGGGCGGAAGAAGTTTCAAACAGGAAAAATAACAACTTAATGACT  
ACTGATGCTGAGTCGCTGCAGgtgtatctttgttacctttactctcttagccttgctgtttctt  
gatataaatttacactgcatagttgtatatctacctcaaaatatgagtccttagatgcaatttacca  
agatagtccttttctgcaactgacgactgaatctgaagcttattctaagattctagaaatcctaa  
gagttgtgattacattttcaacaccttggtcttttgggtgccgttgtaggatttgattttccttta  
tagccaataaacctttaattcgcttgatttgtagaaaaagttacctttgaacagtgttttatc  
taagctcttgcttgaaatcaaagtgtttatctagctgatagctgttcttttccctaacgtttctc  
ttgtgtgtgacagCAAATCTGCCAATCACTTCATACCATGTGGTCGGCGCCATTTTCGTATAATTGT  
AGCACTGGTTCTCCTCTATCAACAATTGGGTGTTGCCTCGATCATTGGTGCAATTGTTTCTTGTCCT  
TATGTTCCCATACAGgttcgtatatcttaataattccccattctcttgcgctgtcggttttttt  
ttccttttgattgcttattttctcatttgcttttcacaccaatgaaaatgattcatttcctccgttt  
at ttggttgaaacagACTGTTATTATAAGCAAAACGCAGAAGTTAACAAAAGAAGGGTTCGACGGT  
ACTGACAAGAGAATTGGCCTAATGAATGAGGTTTTAGCGGCAATGGATACAGTGAAGtacgatact  
ttggaagcctgaaacctaataatttttttcttgcatagttggaagtttggtgagtggtttaactat  
ctcactaaacccaaaatactgtagGTGTTACGCTTGGGAAAAACAGTTTTTCAGTCCAAGGTTCAAAC  
GTACGTGATGATGAATTATCTTGGTTCCGGAAAGCACAACTCCTGTCAGCGgtatggcttgagtgc  
agtgactgttatattaattgattttatagaccgtatgcatgatgtgcatagttgtcttggtcattt  
actgtgcgtctcctaacgggtatgattgtatacaaggacaaatccaagttgctcgtcttttaaat  
gcctttgaccattttgagaatggtatccatcaatatgtgttttaggcattttctgtactattttcra  
gttcattgaacattgattcagttgtttcgggcatgtgtagcagcattcatgcatgatctttaacat  
atattgcattaatgtttctgactcattcttggtcttctatttgctctgcagTTCAATATGTTTATA  
CTAAACAGCATCCCTGTCTCGTGACTGTTGTTTCAATTGGTGTGTTCTCATTGCTTGGAGGAGAT  
CTGACACCTGCAAGAGCGTTTACGTCACTCTCTCTATTTTCTGTGCTTCGCTTCCCTTTATTCATG  
CTTCCAAACATTATAACTCAGgtgatttccttaaaatgtttcttgaaacctgtttcatgtccagt  
actgaataatgtggcatcatagtaattgattgcttctgattgctcttttaattttccatctctacct  
ctttttctagaccagtcggtgtcataatgtttttgcagatgctgaccaggctttacttttgtagAT  
GGTAAATGCTAATGTATCCTTAAACCGTTTGGAGGAGGTACTGTCAACCGAAGAGAGATTCTCTT  
ACCGAATCCTCCCATTTGAACCTGGACAGCCAGCTATCTCAATAAGAAATGGATACTTCTCTGGGA  
TTCAAAGgtcttctttgtctattttatcacatgttcttacttctattagtttctatcattacatat  
tgtcaatgaagtacaaaaagtgagctagaagtatacatatgcagGCGGATAGGCCAACATTGTCAA  
ACATCAACCTGGACATACCTCTTGGCAGCCTAGTTGCGGTAGTTGGCAGCACAGGAGAAGGAAAAA  
CCTCCCTGATATCTGCTATGCTTGGGGAACCTCTGCAAGATCTGATGCGACTGTTACTCTTAGAG  
GATCAGTCGCTTATGTTCCACAAGTTTCATGGATCTTTAACGCAACagtaagtttatatatgctac  
tcagtttatagtatggttctcaatgcgaaaatgtcaaattctcctcttgattgttacttattttg  
tatgtattttatgttttgatatatgatgatgtgtgcttttagatacgtccacatgctgatggttgta  
attaacatcgcgtagGTACGTGACAATATATTGTTTGGGGCTCCTTTTGACCAAGAAAAATATGAA  
AGGGTGATTGATGTGACAGCACTCCAGCATGACCTTGAGTTACTGCCTgtaagtttggtggagagt  
tacttagccatgtgcattgaaaatttctgaggtgaaacgaaccttgaaatctgttggtgcatgt  
aaatcgaaaaaactgaattgcatcagttctgttgatagcatgtacttctattttctagtgtcagg  
tatctaagcttggttctctcttctcttgattgatagGGAGGTGACCTCACGGAGATCGGAGAA  
AGGGGTGTTAACATCAGTGGGGGACAAAAGCAGAGGGTTTCTATGGCTAGGGCCGTTTACTCAAAT  
TCAGACGTGTGCATCTTAGATGAACCATTGAGTGCCCTTGATGCGCATGTTGGTCAGCAGgtaaac  
tagccataggctcttttgatagaacaatactttgtttttcttcaattttgcaaatcgtgaactc  
tataacgttttggttttcaatctgcatggatattctacttcttggttgccacggatctctgccata  
tactacttttaagcaaacattgtttatctgatgttcgaaactggctgttatatatagGTTTTTGAAA  
AATGCATAAAAAGGGAACCTAGGGCAGACAACGAGAGTACTTGTTACAAATCAGCTCCACTTCCTAT  
CACAAGTGGATAAAAATCCTACTTGTCCATGAGGGAACAGTAAAAGAGGAAGGAACATATGAAGAAT  
TATGCCATAGTGGCCCGTTGTTCCCGAGGTTAATGGAAAATGCAGGGAAGGTTGAAGATTATTCCG  
AAGAAAATGGAGAAGCTGAAGTACATCAAACATCTGTAAAACAGTTGAAAATGGGAACGCTAATA  
ATCTGCAGAAGGATGGAATCG

FIGURE 17B  
SUBSTITUTE SHEET (RULE 26)

29/41

AGACAAAGAATTCCAAAGAAGGAAACTCTGTTCTTGTCAAACGAGAAGAACGTGAAACTGGAGTTG  
TGAGTTGGAAAGTCCTGGAGAGGtaagttggcatttcggatTTTTgctctttcttgttgtgttgttg  
cagtattcctttctatcgacagtggaaatatccgtaaataagacatatcttttggttagagcaat  
atgtcaatttatctgtggtgtttctttactacaaaatggatatatattgtttgactcgctctatctc  
atattcatacaaaatgtatatatatattttccgtattaaggttcgtattgtaaagccattgtaataac  
ttgtgaggtgtcaccatgttccagGTACCAGAATGCACCTGGAGGTGCATGGGTAGTGATGATGCT  
CGTTATATGCTACGTCTTGACTCAAGTATTTCCGGTTTCAAGCATCACTTGGTTGAGTGAGTGGAC  
TGATTACAGGAACCCCAAAGACTCATGGACCCCTATTCTATAATATTGTCTATGCGCTTCTTTCGTT  
TGGACAGgtatgagttgcatttggcaaagtgttgagtcggtatcttcatgatcggaataacaatata  
taactgaacattaaaggctgatcagttaagaatatacaccatgtttcttctgcgcaaagtatcga  
gcaaacaaaatggaaaataaaaggatacagagagcaaaacgtttattgctaacacgtatttctgcg  
ggggtttgtcagGTCTCTGTGACATTGATCAATTCATATTGGTTGATTATGTCCAGTCTATATGCA  
GCTAAAAAGATGCATGATGCTATGCTTGGTTCCATACTAAGGGCTCCAATGGTGTCTTTCAAACC  
AATCCATTAGGACGGATAATCAATCGATTGCAAAAGATATGGGAGATATTGATCGAACTGTGGCA  
GTCTTTGTAAACATGTTTATGGGTTCAATCGCACAGCTTCTTTCAACTGTTATCTTGATTGGCATT  
GTCAGCACTCTGTCCCTGTGGGCCATCATGCCCTGTTGGTCTGTCTATGGAGCTTATCTGTAT  
TACCAGtgtaacctacataactttttaaacgcaatgctatctacattcatgactacagatcgagaca  
tggaaaactgagaccaaaaaggaacactgattgtgtcatatctgttgtgtcataacctgatttttcc  
ttattgtagAACACATCTCGGGAATTAAACGTATGGATTCCACTACAAGATCGCCAGTTTATGCT  
CAATTTGGTGAGGCATTGAATGGACTATCTAGTATCCGTGCTTATAAAGCATATGACAGGATGGCT  
GAAATTAATGGAAGGTCAATGGACAATAACATCAGATTCACACTTGTAACATGGCTGCAAATCGG  
TGGCTGGGAATCCGTTTGGAAAGTTTTGGGAGGTCTCATGGTTTGGTGGACTGCTTCATTAGCCGTC  
ATGCAGAACGGAAGGCAGCGAACCAACAAGCATATGCATCTACGATGGGTTTGGCTTCTCAGTTAT  
GCGTTAAGCATTACCAGCTCTTTAACAGCTGTACTGAGACTCGCGAGTCTAGCTGAGAATAGTTTA  
AACTCGGTTGAGCGTGTGGAAATTATATCGAGATACCATCAGAGGCTCCATTGGTCATTGAAAAC  
AACCGTCCACCTCCCGGATGGCCATCATCTGGATCCATAAAATTTGAGGATGTTGTTCTTCGTTAC  
CGCCCTGAGTTACCTCCTGTTCTTCATGGAGTTTCGTTCTTGATTCTCCAATGGATAAGGTGGGA  
ATTGTTGGGAGGACAGGCGCTGGGAAATCAAGCCTCTTAAATGCCTTATTCAGGATTGTGGAGCTG  
GAAAAAGGAAGGATTTTAATTGATGAATGCGACATTGGAAGATTTGGACTGATGGACCTACGTAAA  
GTGGTCGGAATTATACCGCAAGCGCCAGTTCTTTTCTCAGGTACCGTGAGATTCAATCTTGACCCA  
TTTAGTGAACACAACGACGCCGATCTCTGGGAATCTCTTGAGAGGGCACACTTGAAAGATACTATC  
CGCAGAAATCCTCTTGGTCTTGATGCTGAGgtacttaattaaatatttccatttgggaaagtctca  
tgtattcagtaataataactcagtccttttgggtcagGTAAGTGAAGCAGGAGAGAATTTAGTGT  
GGACAGAGACAGTTGTTGAGTCTTGACCGTGCAATTGTTACGAAGATCTAAGATACTTGTCTTGAT  
GAAGCAACTGCTGCAGTTGACGTAAGAACTGATGTTCTCATCCAAAAGACCATCCGAGAAGAATTC  
AAGTCATGCACAATGCTAATCATCGCTCATCGTCTCAATACTATCATCGACTGTGACAAAGTTCTT  
GTCCTTGATTCTGGAAAgtacgtatacaaaaatattcgaccactacttgcatcaatttaactactt  
ttgagctaacatatattgagattcccaacacctcagGTTACAGGAATTCAGTTACCCGGAGAATCTT  
CTTTCAAATGGAGAAAGTTCTTTCTCGAAGATGGTTCAAAGTACAGGAAGTCAAACCGCGGAGTAC  
TTACGTAGTATAACACTAGAGAACAACCGTACCAGAGAAGCTAACGGTGATGATTACAACTTTA  
GAAGGTCAAAGGAAATGGCAAGCTTCTTCTCGTTGGGCTGCAGCTGCTCAATTTGCATTGGCTGTG  
AGCCTCACTTCATCTCACAACGACCTCCAAAGCCTTGAAATCGAAGATGATAACAGTATTTTGAAG  
AAAACAAAGGACGCCGTCGTCACCTTACGCACTGTCCTTGAAAGGAAACATGATAAAGAGATTGAA  
GACTCTCTAAACCAAAGTGACATCTCTAGAGAGCGTTGGTGGCCATCTCTTACAAAATGGTTCGAA  
Ggtaacgttattcttaagatttctgatacaggtatacagacataaagaattgttgaagtttcttgat  
ctaataatttgtgtatataactctcagGGCTTGCCGTGATGAGCAGATTGGCGAGGAACAGAATGCA  
ACACCCGATTACAATTTAGAAGGGAAATCGTTTGAAGTGGGACAATGTCGAGATGTAAacgatgaa  
aggcttacactaatagacctaaaactcccattttgatggaaacttttatttgtattgcttgggatac  
acgtaacaaaatgccattaatcgtggtgtaactatataggctatgcttcttttgggaaaaagaga  
gtttgattacagaggatgtgatgataacacaattggaattcaaatttgcagcaaaatttgggagaa  
aaaaaaaagtcaatgagtgcaacatgcc

FIGURE 17C

SUBSTITUTE SHEET (RULE 26)

30/41

aacatggtttcaacttctggacatggacaaccattggacataatttctctcacaggaccatgtttt  
gtcattgacattttgcacaaaaatgttctattaaacatatatctataaagaatttgaacaattgtt  
aaaa aaacacttaaaatataaattgcaatacaaatttcctttttt

FIGURE 17D

SUBSTITUTE SHEET (RULE 26)



MGFEPLDWYCKPVPNGVWTKTVDYAFGAYTPCAIDSFVLGISHLVLLILCLYRLWLITKD  
HKVDKFCLRSKWFSYFLALLAAYATAEPLFRLVMRISVLDLDGAGFPPYEAFLVLEAFA  
WGSALVMTVVETKTYIHELWYVRFVAVIYALVGDMLLNVLVSKEYYGSFKLYLYISEV  
AVQVAFGTLLFVYFPNLDPPYGYTPVGTENSEDYEEELPGGENICPERHANLFDSIFFS  
WLNPLMTLGSKRPLTEKDVWHLDTWDKTETLMRSFQKSWDKELEKPKPWLLRALNNSLGG  
RFFWGGFWKIGNDCSQFVGPLLLNELLKSMQLNEPAWIGYIYASIFVGVVLGVLCEAQY  
FQNMVRVGYRLRSALIAAVFRKSLRLTNEGRKKFQTKITNLMTTDAESLQQICQSLHTM  
WSAPFRIIVALVLLYQQLGVASIIGALFLVLMFPPIQTVIISKTKLKEGLQRTDKRIGL  
MNEVLAAMDTVKCYAWENSFQSKVQTVRDELSWFRKAQLLSAFNMFILNSIPVLVTVVS  
FGVFSLLGGDLTPARAFTSLSLFSVLRFLFMLPNIITQMVNANVSLNRLEEVLSTEERV  
LLPNPPIEPGQPAISIRNGYFSWDSKADRPTLSNINLDIPLGSLVAVVGSTGEGKTSLS  
AMLGELPARSDATVTLRGSVAYVPQVSWIFNATVRDNILFGAPFDQEKYERVIDVTALQH  
DLELLPGGDLTEIGERGVNISGGQKQRVSMARAVYSNSDVCILDEPLSALDAHVGQOVFE  
KCIKRELQOTTRVLVTNQLHFLSQVDKILLVHEGTVKEEGTYEELCHSGPLFPRLMENAG  
KVEDYSEENGAEVHQTSVKPVENGANNLQKDIETKNSKEGNSVLVKREERETGVVSW  
KVLERYQNALGGAWVVMMLVICYVLTQVFRVSSITWLSEWTDSGTPKTHGPLFYNIYAL  
LSFGQVSVTLINSYWLIMSSLYAAKKMHDAMLGSILRAPMVFFQTNPLGRIINRFAKDMG  
DIDRTVAVFVNMFMGSIAQLLSTVILIGIVSTLSLWAIMPLLVFYGAYLYYQNTSREIK  
RMDSTTRSPVYAQFGEALNGLSSIRAYKAYDRMAEINGRSMDDNNIRFTLVNMAANRWLGI  
RLEVLGGLMVWWTASLAVMQNGKAANQQAYASTMGLLLSYALSITSSLTAVLRLASLAEN  
SLNSVERVGNYIEIPSEAPLVIENNRPPPGWPSGSIKFEDVVLRYRPELPPVLHGVSF  
ISPMDKVGIVGRTGAGKSSLLNALFRIVELEKGRILIDECDIGRFLMDLRKVVGIIIPQA  
PVLFSGTVRFNLDPFSEHNDAD...ESLERAHLKDTIRRNPLGLDAEVTEAGENFSVGQRQ  
LLSLARALLRRSKILVLDEATAAVDVRTDVLIQKTIREEFKSCTMLIIAHLNTIIDCDK  
VLVLDSGKVQEFSSPENLLSNGESSFSKMVQSTGTANAAYLRSITLENKRTREANGDDSQ  
PLEGQRKWQASSRWAAAQFALAVSLTSSHNDLQSLIEDDNSILKKTDAVVTLRVLE  
GKHDKEIEDSLNQSDISRERWWPSLYKMVEGLAVMSRLARNRMQHPDYNLEGKSFWDWNV  
EM

FIGURE 18

SUBSTITUTE SHEET (RULE 26)

32/41

Fig. 19A

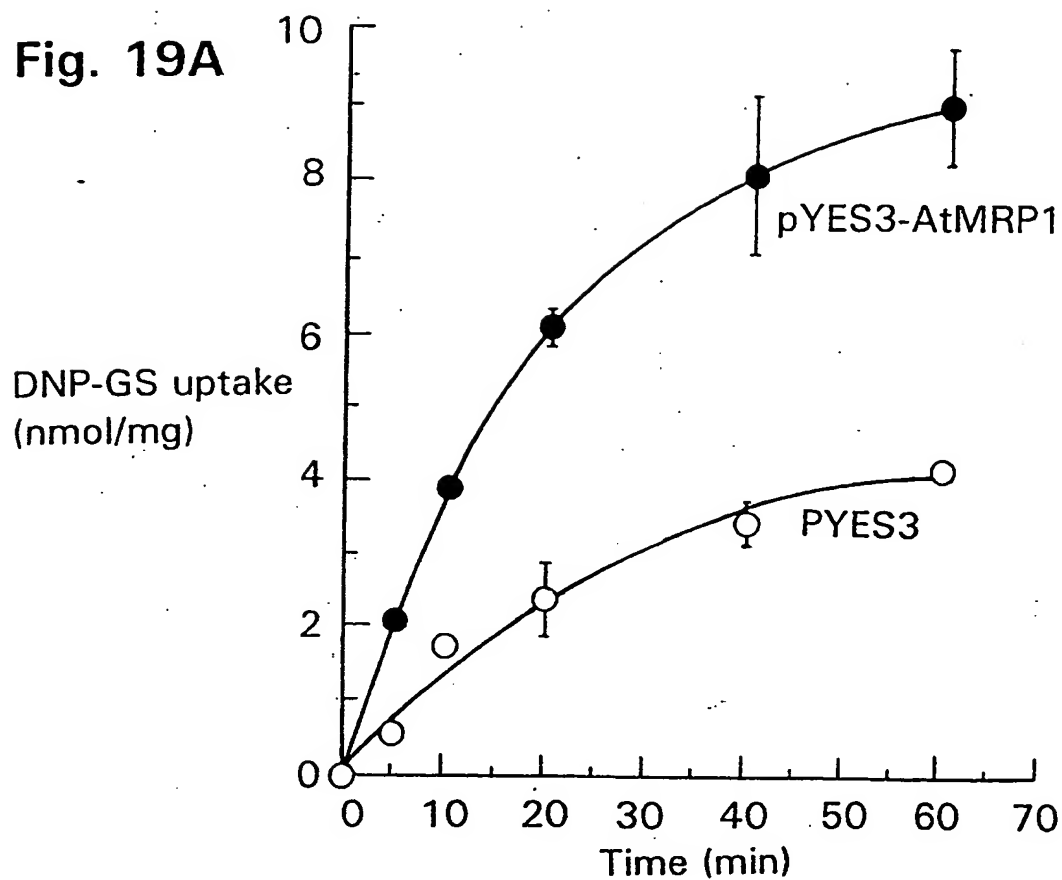
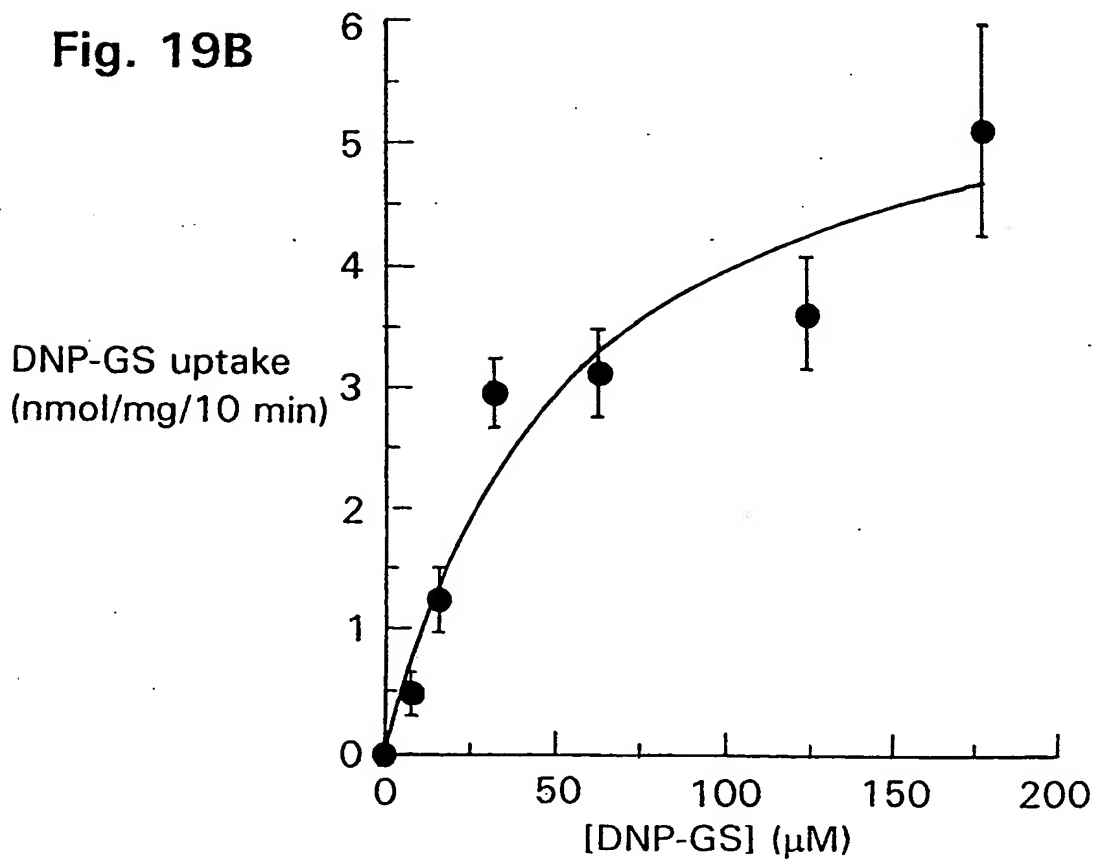


Fig. 19B



SUBSTITUTE SHEET (RULE 26)

33/41

Fig. 20A

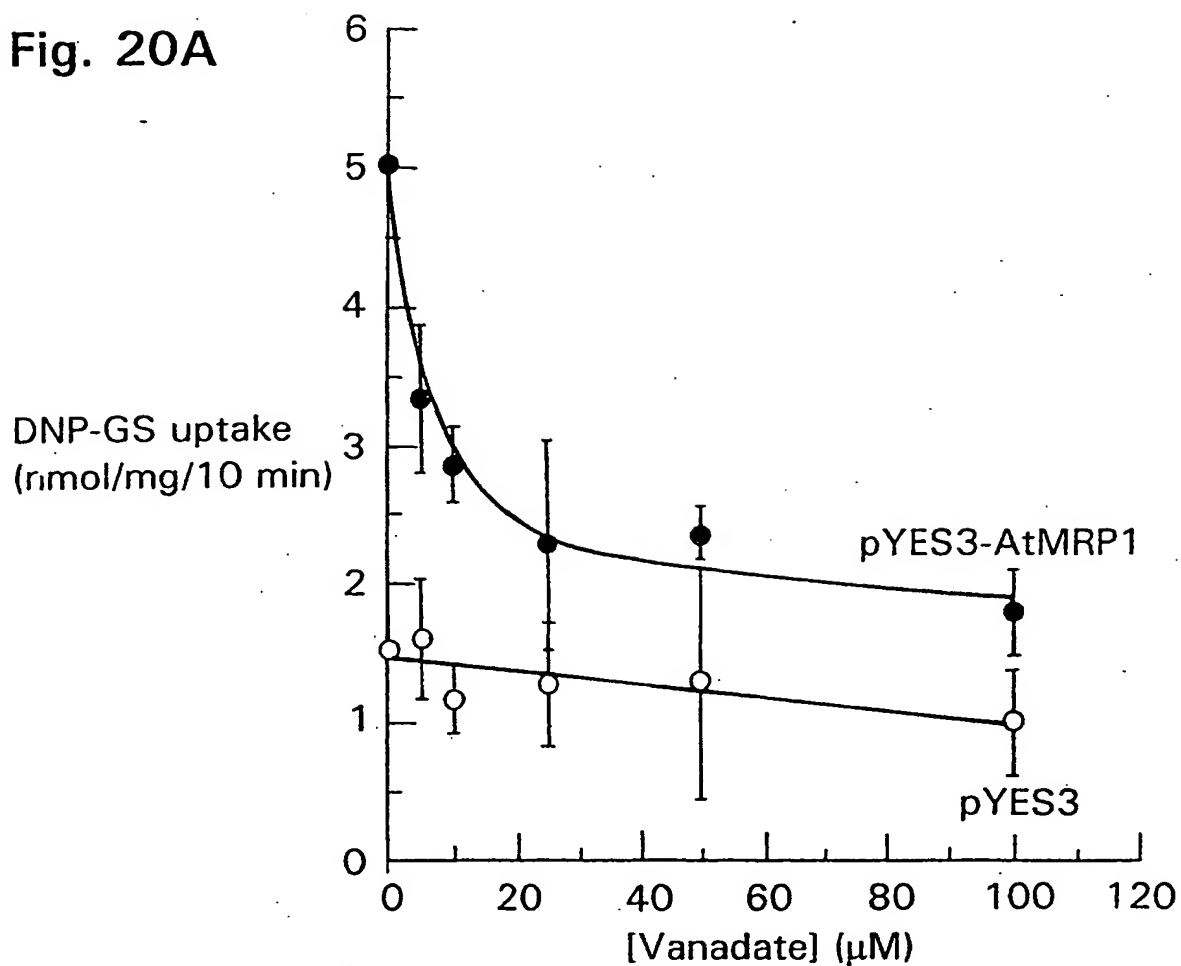
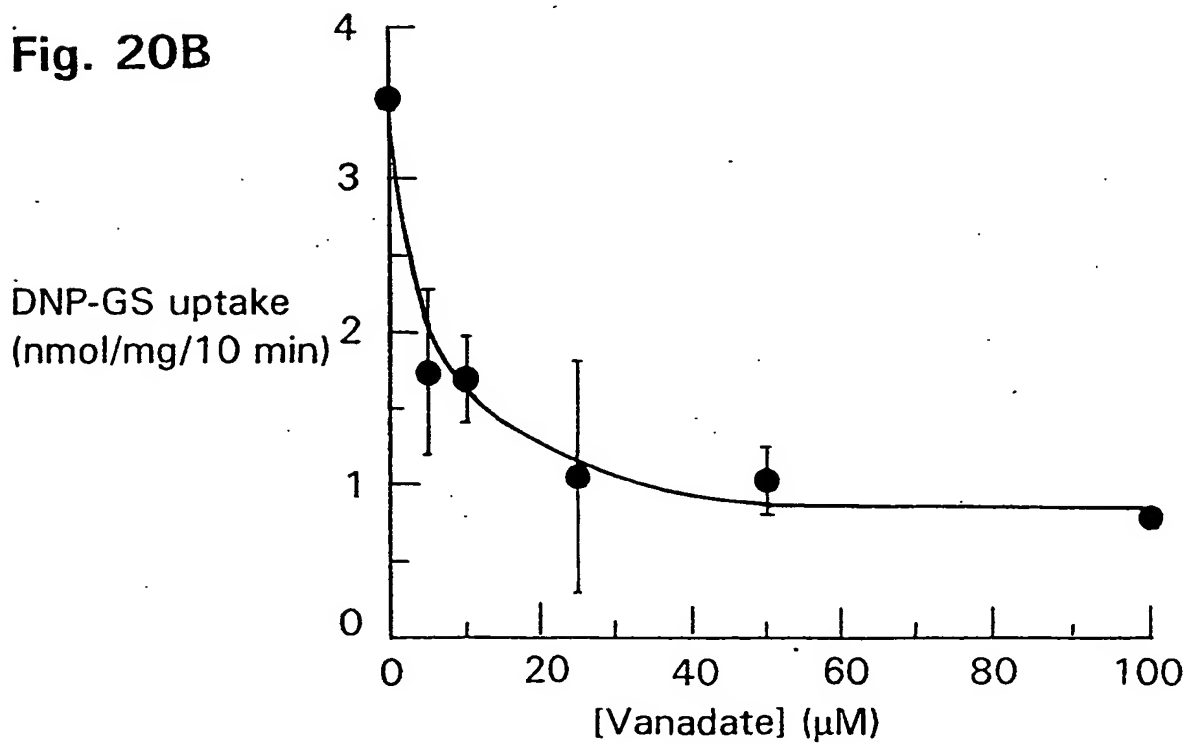


Fig. 20B



SUBSTITUTE SHEET (RULE 26)

34/41

Fig. 21

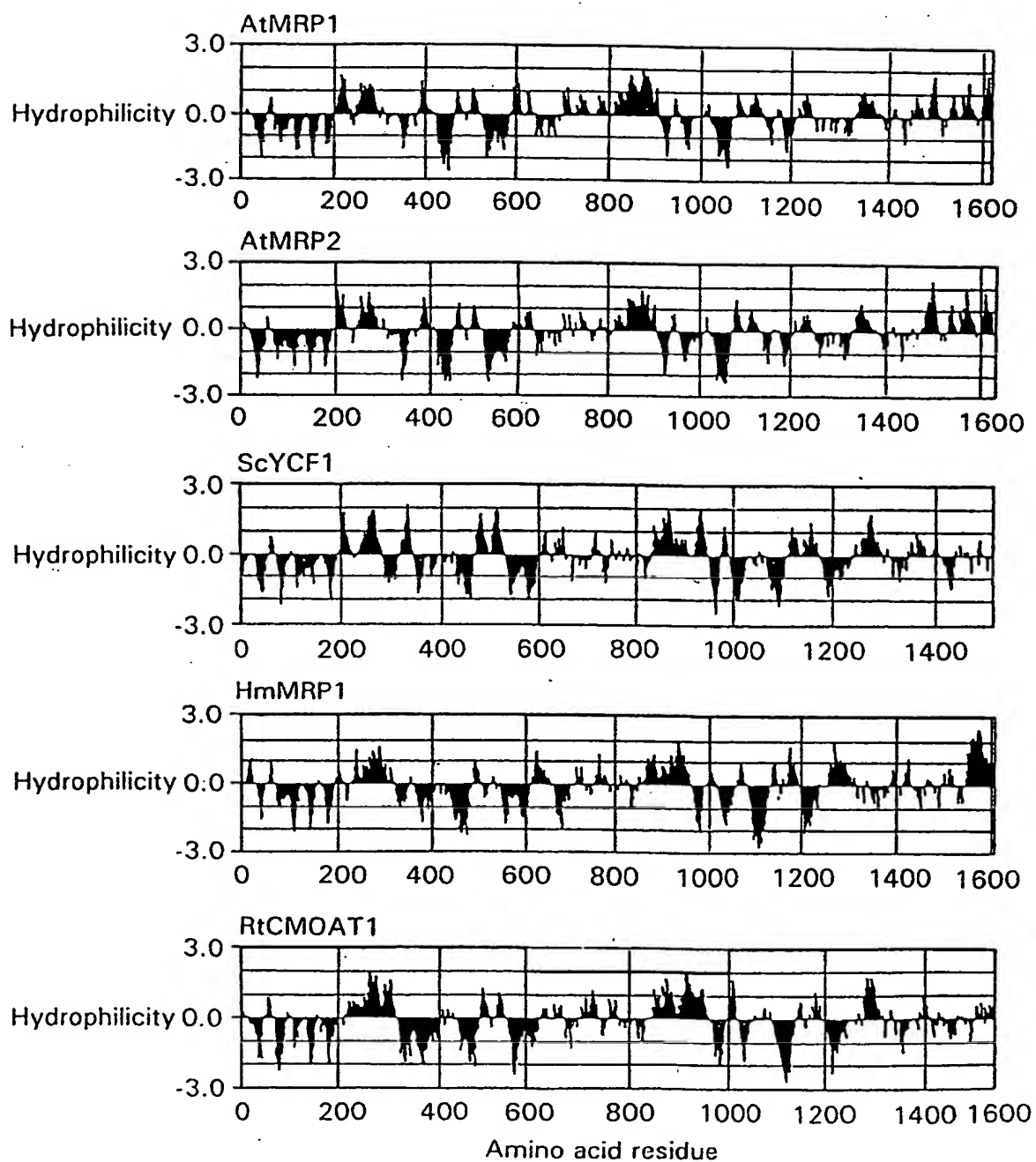
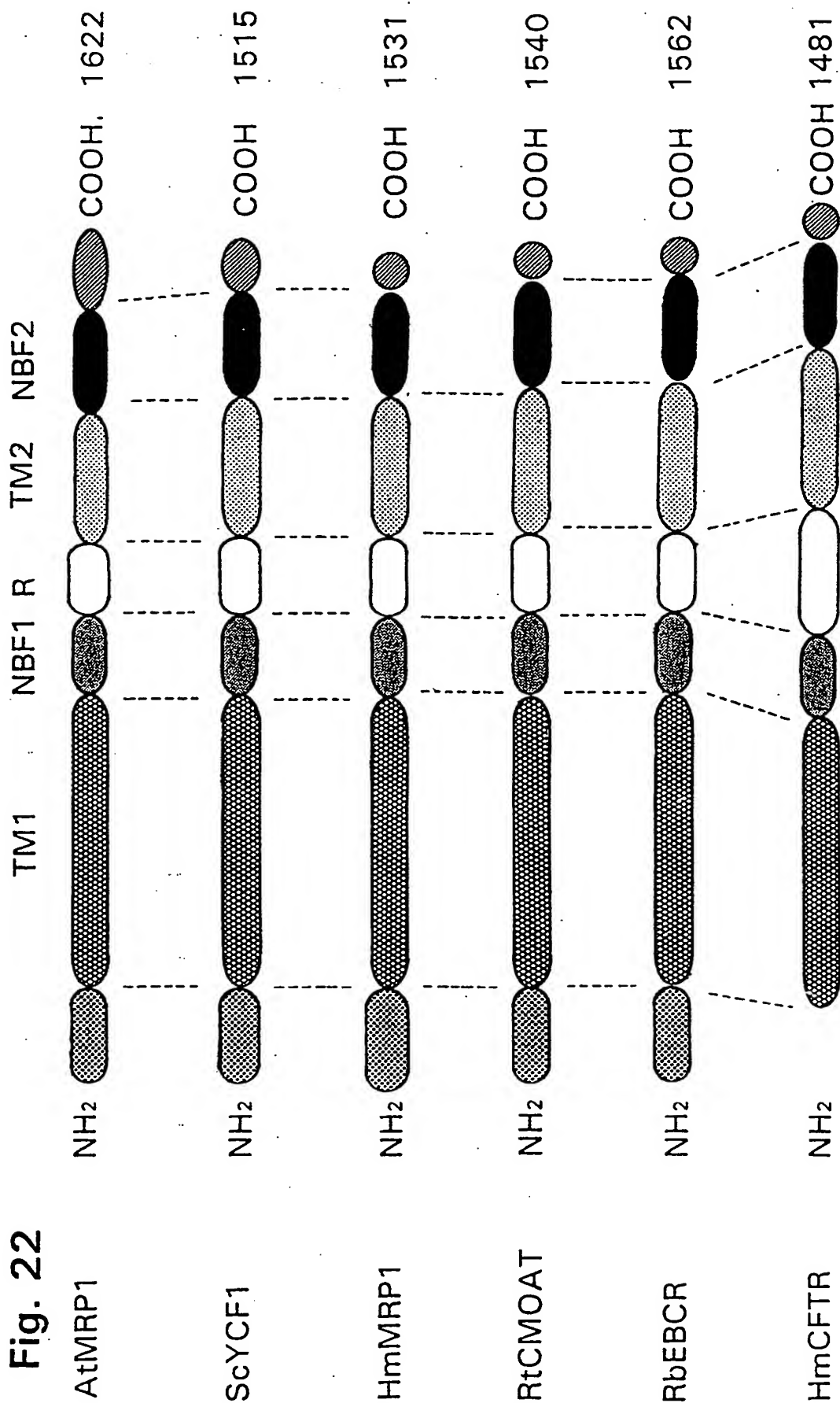


Fig. 22



AtMRP1 Promoter Sequence: 1253 bpContains:

- 2 Myb recognition sequences (atatacggtta and agttagtt).
- 1 xenobiotic regulatory element (GCGTG).
- 1 antioxidant response element (Gtgacaaa).

NB: Several "RNA instability determinants" (ATTTA) found but these usually located in the 3'-UTRs of genes? May simply be reflection of AT-richness of sequence.

ttcacttttgtccttttttttcttaacatctacttttgtcatcagcaaattatctgtaaataa  
gataggggttatgcttattgctacaatgaacctaatcctatgatgtgtattgcaatttgcaa  
ccatgcgagtttaattatttgtttactgctatagtgatcattttatgatgtgtttttattaa  
ttacaaaacagagcatcaaaaatcaaaagaacatatcgcataatcgaaactatgctaatacct  
ctcctcaatctttgttgttgttatattcaagtagcttattcttttgtttttattttacgatta  
gatttctctagaATTTAATTTAatattATTTAatcatacttgatcaagggtttagcttaatc  
aatatcggttatcggtgcatcctgcagattcaaatgatcaagtcataataatctacttatatgt  
attatatatattagataaccaccaacgaaacaaaatcatatttctataacatttgtttgggta  
aatatATTTAagatttgttaacagttgttcgggttcaaaactatcactttgtagttgtagga  
tgaggaaaagtcgtgatgatcatctactaaaatcatgtgttttttaagaacatgatttt  
cattggatagtttaataaatgttaaaaaaataactaagtgtcaaagaagagatttgaaccata  
tgtagaataacttgattcgaatttttctctgacgaataatctaatactcttttctcaaaagaaa  
aaaatgtttgttaacttggacacgatattattatccaacttctttctagatattcattttt  
aaattacctatataatttttattttctcaaaatataactaaaaattggatagagctattaaata  
aaaaagatagaATTTAagagagaaatagcaacataatgaattataataataatattttgtaaa  
gaaataacaaactttatagttagtttgcctaatatagaaaaaagatacagttATTTAacccat  
ttgtttgtgtgttaaaaaaaggagtaaaataaacagagaaaaagagcttcttgtttttacttgt  
gaacgttattgacttttcggcctctctctctctctctatacaaatatatggatcttcatttct  
tcgtatagtgtgaagcagtgacgcacatccATTTAatcatctctcttataaatctcgaatctgc  
cacagagagaGCGTGtgacaaaatgagttcataagattccgttatcgtcttctctgattcctc  
caaactctccgg

**FIGURE 23**

37/41

AtMRP2 Promoter Sequence: 1368 bpContains:

1 bZIP recognition sequence (**cacgtg**).  
Xenobiotic regulatory element not found.  
Antioxidant response element not found.

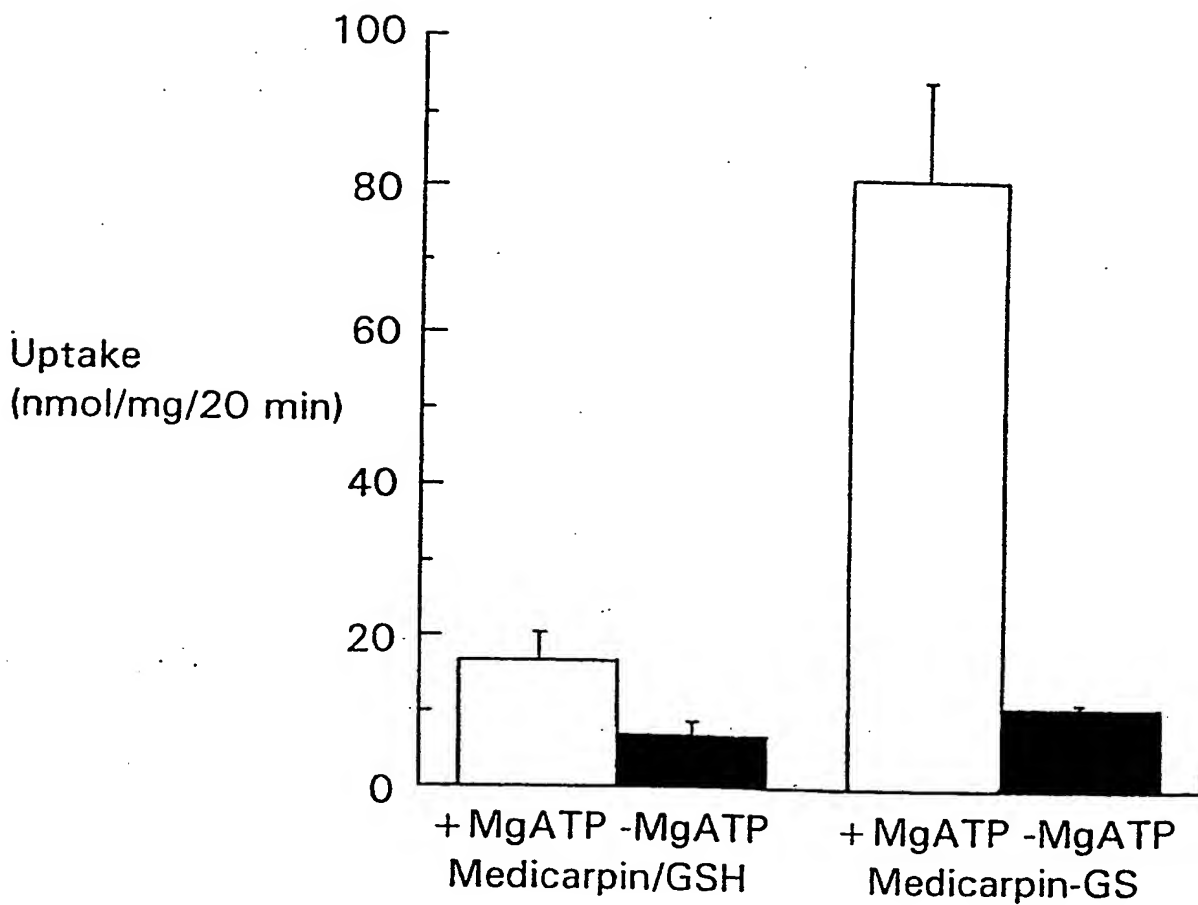
NB: Several "RNA instability determinants" (ATTTA) found but these usually located in the 3'-UTRs of genes? May simply be reflection of AT-richness of sequence.

```
aaacaattggtgtatTTTTgaatttttcatgcaacgccacgtgaacagcttaattgcttgatt
ggaacaaacctTTTTtagaattcattaatcagtttttaggtgttttgaaaaattaacgaact
atagtggagattaattaattttatattagtccttttttagtacacaaatcgaagtttcctag
atTTTTcaaagttgaaaataatattgataatATTTAtcaacaatgaatctacaaaaacat
aattTTTTtgccaaacaataacaccgaaacaagattcattcactatTTTTggtttaaaaa
aaaaaatcaaaattacactattatgaagccaattTTTTgtatgcaaaaaacctgtatgtatc
aatttgTTTTgtattaaaaagtaagcATTTAtgtctTTTTtataaataatagaaacactt
actagatgaatagattTTTTggttttagaacagaataactataattgtATTTAtatagcttt
tttatattattcgatatagaaaagtgttataataggaaaaatgtaccatatactgtcaata
acatatttgattctaaatataaatagaattgttttaaagaaatgatcggtttataattaa
atgggtttttaatgtcttttcttggggcaaaaaacaaagcttgtctttcgtccatatatttg
catcgtaaggggtgacgtatcactctctctttctctcaaatattattcttcaatctctttt
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ctcgttggtgattgcttgctcactgttctatctttatatatgattaaatgcaattttgccct
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acataatgattgATTTAtgacttttttaaagttatgaaaATTTAtattttttgttgctatgg
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tgtggtagcttgatgcactgtgtt
```

FIGURE 24

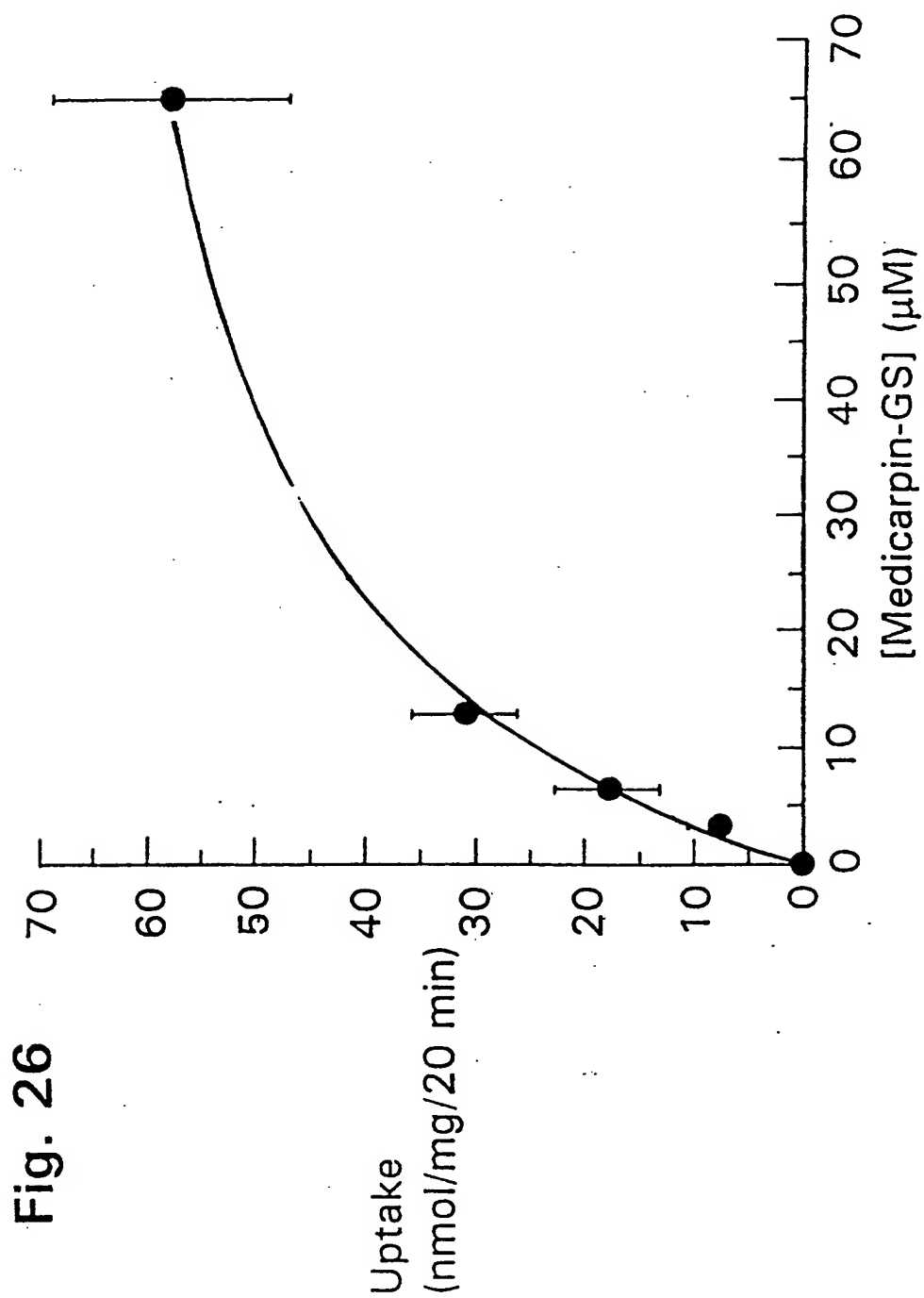
38/41

Fig. 25





39/41



40/41

Fig. 27A

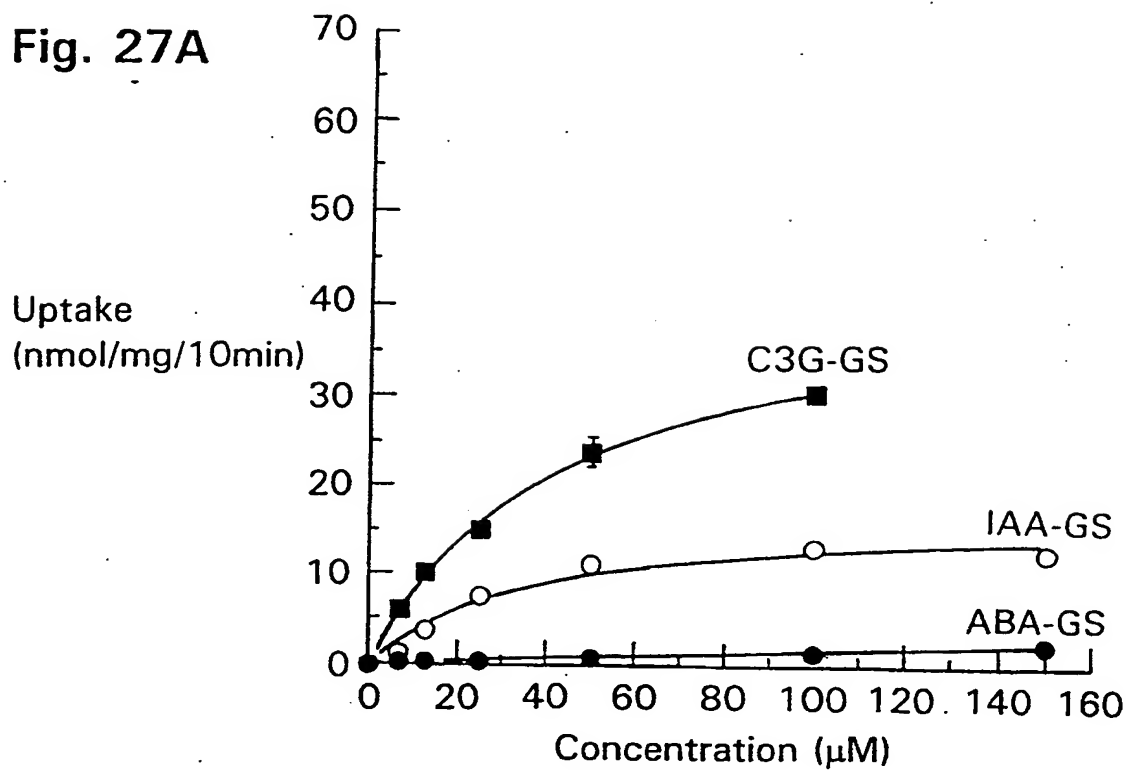
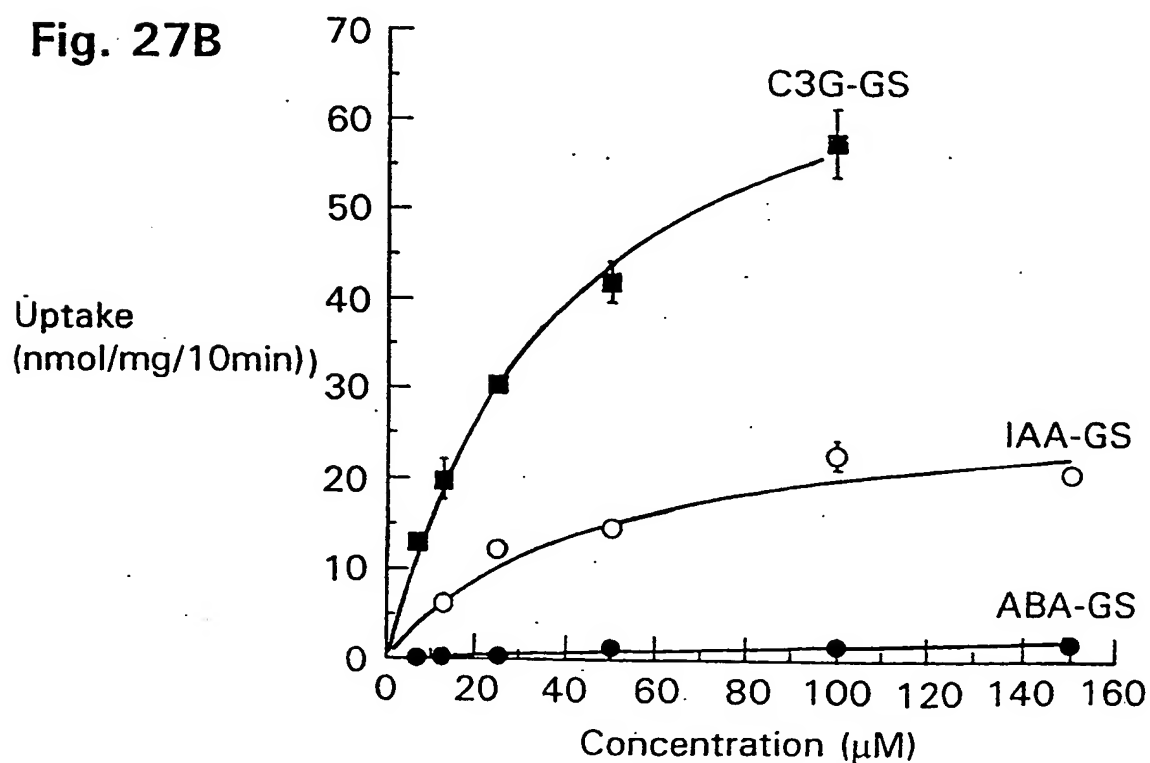


Fig. 27B



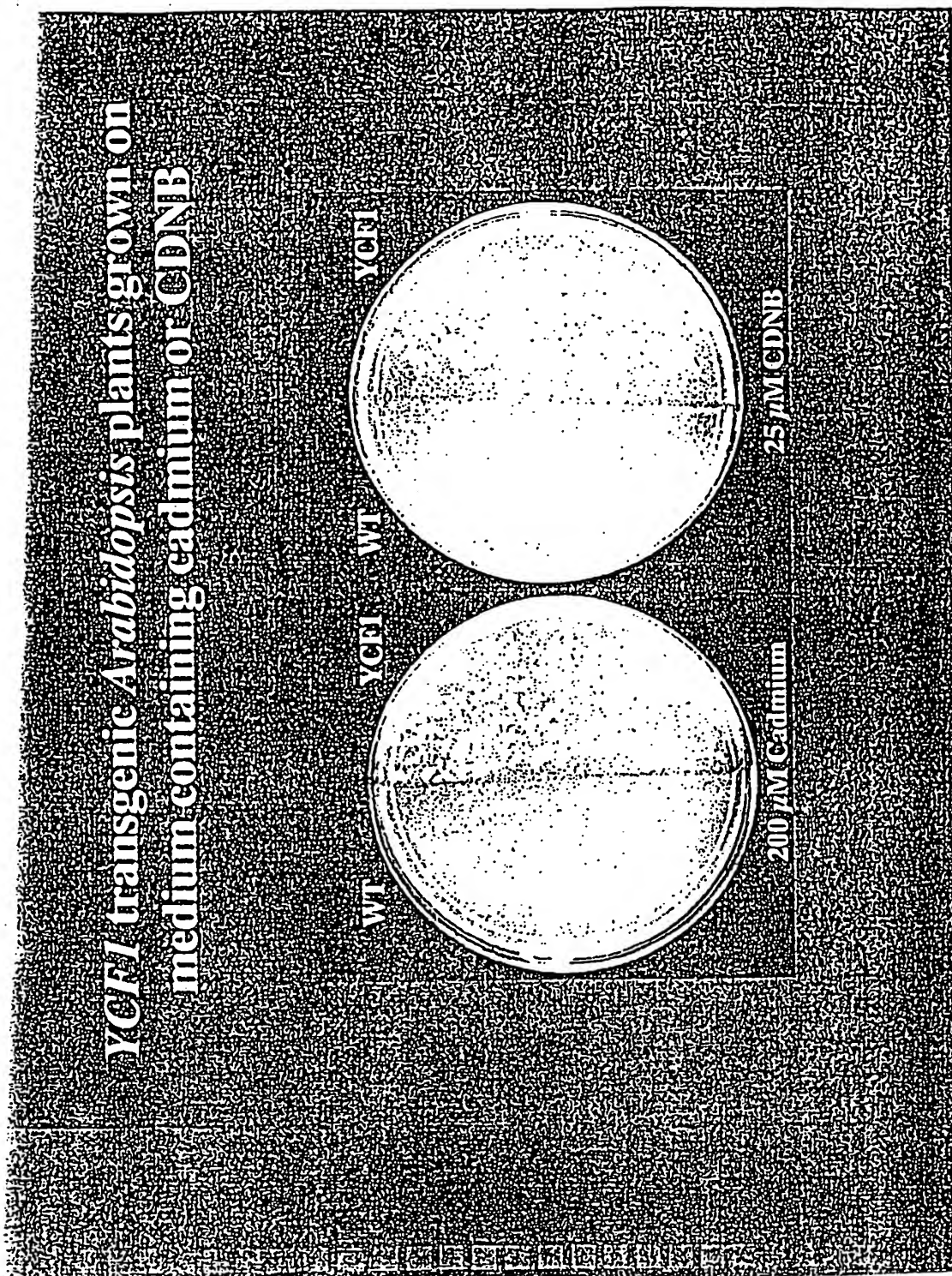


FIGURE 28

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/21336

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.6, 23.1, 24.1; 530/350, 387.9; 435/252.3, 419, 172.3, 6; 800/205

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
APS, DIALOG - Biotech files

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	ISHIKAWA, T. et al. The GS-X Pump in Plant, Yeast, and Animal Cells: Structure, Function, and Gene Expression. Bioscience Reports. 1997. Vol 17, No. 2, pages 189-207, see entire document.	1-27
A	LI, Z.S. et al. Magnesium Adenosine 5'-Triphosphate-Energized Transport of Glutathione-S-Conjugates by Plant Vacuolar Membrane Vesicles. Plant Physiol., 1995, Vol 107, pages 1257-1268, see entire document.	1-27

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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*O* document referring to an oral disclosure, use, exhibition or other means	
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Date of the actual completion of the international search

26 JANUARY 1998

Date of mailing of the international search report

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/21336

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	LI, Z.S. et al. A new pathway for vacuolar cadmium sequestration in <i>Saccharomyces cerevisiae</i> : YCF1-catalyzed transport of bis(glutathionato)cadmium. <i>Proc. Natl. Acad. Sci. USA</i> , January 1997, Vol 94, pages 42-47, see entire document.	1-27
T	LU, Y.P. et al. AtMRP1 gene of <i>Arabidopsis</i> encodes a glutathione S-conjugate pump: Isolation and functional definition of a plant ATP-binding cassette transporter gene. <i>Proc. Natl. Acad. Sci. USA</i> . July 1997, Vol 94, pages 8243-8248, see entire document.	1-27
A	MARTINOLA, E. et al. ATP-dependent glutathione S-conjugate 'export' pump in the vacuolar membrane of plants. <i>Nature</i> , 15 July 1993, Vol 364, pages 247-9249, see entire document.	1-27
A	LI, Z.S. et al. The Yeast Cadmium Factor Protein (YCF1) Is a Vacuolar Glutathione S-Conjugate Pump. <i>J. Biol. Chem.</i> , 15 March 1996, Vol 271, No. 11, pages 6509-6517, see entire document.	1-27

Form PCT/ISA/210 (continuation of second sheet)(July 1992)\*

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/21336

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

A01H 5/00; C07K 14/415, 16/16; C12N 1/13, 1/21, 5/10, 15/29, 15/64, 15/82

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

536/23.6, 23.1, 24.1; 530/350, 387.9; 435/252.3, 419, 172.3, 6; 800/205

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